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Optimizing Fecal Microbiota Transplantations For Therapeutic Use Beyond Clostridioides difficile Infection

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Abstract

Fecal microbiota transplantation (FMT) is an emerging and effective therapy for the treatment of recurrent *Clostridioides difficile* infection. Members of the gut microbiome have been implicated in other diseases and FMT has been considered as a potential therapy. Two such conditions include non-alcoholic fatty liver disease (NAFLD) and multiple sclerosis (MS), both of which involve increased small intestinal permeability believed to contribute to the development and disease progression. One of the aims of this project was to determine if FMT could be safely used in patients with NAFLD and MS to improve health outcomes. Before starting the clinical studies, optimal ways of storing and preparing stool for FMT were investigated with a goal of reducing loss of viable bacteria due to sample collection, handling, and storage. Bacterial culture and next-generation sequencing techniques were utilized to assess the impact of processing and storage. With optimal procedures in place, which included storing samples as whole stool at -80 °C for up to 3 months, FMT donor screening was expanded to extend beyond transmissible diseases to include lifestyle factors, and personal/family history of disease. From this, only 5 of 46 healthy potential donors qualified, and they provided stool for patients with NAFLD (n=21) and MS (n=10). All FMT recipients with elevated small intestinal permeability, determined using the lactulose:mannitol permeability assay, improved following FMT. Microbiota engraftment was detected in some patients. The treatment was safe and well tolerated in all recipients. With NAFLD being the second leading cause of liver transplant in North America and MS having no cure, the use of FMT could potentially contribute to the quality of life and reduction in comorbidities. Since current treatment options for both diseases are not particularly effective, and the rates are increasing, new approaches are needed. The current findings provide a basis for larger studies with earlier intervention and longer follow-up. In summary, the improvement in intestinal barrier function with FMT shows a novel mechanism for this therapy, and one that has implications for many conditions associated with abnormal intestinal permeability.

Keywords

Fecal microbiota transplant, *Clostridioides difficile* infection, metabolic syndrome, non-alcoholic fatty liver disease, multiple sclerosis, gut, microbiome, microbiota, intestinal permeability, butyrate.

Summary for Lay Audience

Fecal microbiota transplantation (FMT) is the transfer of stool from a healthy donor into the intestine of a diseased recipient. This requires stool samples to be homogenized and filtered, and the end product results in the transfer of microorganisms, including bacteria, viruses, archaea, and fungi, plus undigested food and fibre, and host cells into the intestine of the recipient. FMT has effectively treated recurrent *Clostridioides difficile* infections that cause severe and prolonged diarrhea in patients, but a number of other conditions may also benefit from these transplants. The first goal of this thesis was to ensure that storage and handling of donor samples optimally retained the viability of bacteria, which are likely a necessary component of FMT to be effective. Selecting donors for FMT is not simple, as many healthy people may be carriers of infectious diseases and/or have a person/family history of disease that could be passed along by FMT. Surprisingly, only 1 in 10 healthy people qualified to be a stool donor using our criteria. Using fecal material from the selected donors, two clinical trials were undertaken, which was the second goal of this thesis. The purpose of these two studies was to treat patients with non-alcoholic fatty liver disease (NAFLD) and others with multiple sclerosis (MS). Both of these conditions share having a modified bacterial composition in the gut, compared to healthy people. In addition, both have abnormal gut barrier function or "leaky gut syndrome", which means that the proteins that usually bind intestinal epithelial cells tightly together to stop microorganisms and small molecules from entering the bloodstream, are less prevalent and less effective. The administration of FMT restored gut barrier function in these studies. The diseases NAFLD and MS are examples of chronic illnesses that afflict people worldwide. While there are a number of therapies available for both diseases that seek to reduce the symptoms or treat disease, none are particularly efficient, and the rates of both these diseases are slowly increasing. As more successful FMT donors are characterized and FMT becomes more readily available, we may see expanded uses of this therapy for a variety of other conditions that have links to abnormal gut barrier function.

Co-Authorship Statement

Chapter 2: This chapter of my thesis has been submitted for publication and is currently under revision (**Laura J. Craven**, Gregory B. Gloor, Gregor Reid, Seema Nair Parvathy, Michael Silverman, and Jeremy P. Burton. Commonly used methods to prepare fecal samples for fecal microbiota transplantation results in decreases in bacterial viability.). I was the lead author of the manuscript. I was involved in study design, methodology development, collection of data, analysis of data, and writing of the manuscript. GBG, GR, MS, and JPB were involved in experimental design. GBG reviewed the data for Figures 2.5, 2.6, and 2.7. All authors reviewed and revised the manuscript.

Chapter 3: This manuscript is currently in progress for submission for publication (**Laura J. Craven**, Da In Lee, Gregory B. Gloor, Kait F. Al, Gregor Reid, Seema Nair Parvathy, Michael Silverman, Jeremy P. Burton. Characterization of viable bacteria throughout preparation and storage of fecal microbiota transplantation capsules.) I am the lead author of the manuscript. I was involved in study design, methodology development, collection of data, analysis of data, and writing of the manuscript. GBG, GR, MS, and JPB were involved in experimental design. DIL helped acquire viability data. KFA extracted and amplified the DNA for next-generation sequencing. GBG wrote custom R scripts to demultiplex and filter the sequencing data. All authors reviewed and revised the manuscript.

Chapter 4: This manuscript was published in *Open Forum Infectious Diseases* (**Laura J. Craven**, Seema Nair Parvathy, Justin Tat-Ko, Jeremy P. Burton, and Michael Silverman. Extended screening costs associated with selecting donors for fecal microbiota transplantation for treatment of metabolic syndrome-associated diseases. *Open Forum Infectious Diseases*. **4**; ofx243 (2017). doi: 10.1093/ofid/ofx243). I was the lead author of the manuscript. MS and SNP recruited and screened the potential FMT donors. I was involved in analyzing data collected by MS, SNP, and JT, collecting data on the various donor screening methods used by different FMT clinics, and writing the manuscript. All authors reviewed and revised the manuscript.

Chapter 5: This manuscript has been accepted for publication at *The American Journal of Gastroenterology*. (**Craven LJ**, Rahman A, Parvathy S, Beaton M, Silverman J, Qumosani K, Hramiak I, Hegele RA, Joy T, Meddings J, Urqhart BL, Harvie R, McKenzie CA, Summers

K, Reid G, Burton JP and Silverman M. Allogenic fecal microbiota transplantation in patients with non-alcoholic fatty liver disease improves abnormal small intestinal permeability: a randomized control trial). I was the lead author of the manuscript. I was involved in study design, collection of data (with the exception of MRI and intestinal permeability data), analysis of data (with the exception of MRI and intestinal permeability data), and writing of the manuscript. AR, SP, MB, IH RAH, JT, JM, BLU, RH, CAM, SK, GR, JPB, and MS were involved in study design. AR performed the FMTs. LJC and SP prepared FMT material. JS was involved in microbiome analysis. MB and KQ recruited and assessed patients. JM analyzed urine for intestinal permeability. RH administered dietary history recall. CAM analyzed PDFF data. All authors reviewed and revised the manuscript.

Chapter 6: This manuscript is currently in progress for submission for publication. I am the lead author of the manuscript. Other authors involved include Ana Wing, Chantelle Graf, Kate Parham, Steve Kerfoot, Seema Nair Parvathy, Jeremy P. Burton, Marcelo Kremenchutzky, Sarah Morrow, Courtney Casserly, and Michael Silverman. I was involved in study design, methodology development, FMT preparation, collection of data (with the exception of blood samples, MRI, and intestinal permeability data), analysis of data (with the exception of MRI data) and writing of the manuscript. MK and AW recruited, interviewed and assessed patients. CG collected blood and urine samples, assessed patients, and administered FMTs. KP and SK analyzed cytokine data (data not included in thesis). AW, KP, SK, SP, JPB, MK, and MS were involved in study design. SM and CC reviewed MRI data (data not included in thesis).

Dedication

I would like to dedicate this thesis to Dr. Tamara Spaic, Annie Kudirka, and the rest of my incredible team at the Endocrinology Clinic and Diabetes Education Centre at St. Joseph's Health Care, without whom I would not have been able to overcome and manage my diagnosis of type 1 diabetes during my PhD. Thank you!

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Finally, I would like to dedicate this thesis to my grandmother, Joan McNulty (January 7th, 1935-July 25th, 2017). She was always an inspiration to me as the first woman in my family to attend university and she dedicated her life to teaching others. She was always my biggest cheerleader in pursing my academic dreams, and for that I am eternally grateful.

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List of Abbreviations

ALDEx	ANOVA-like differential expression
Alk Phos	Alkaline phosphatase
ALT	Alanine aminotransferase
Anti-tTG	Transglutaminase 2 antibodies
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
AST	Aspartate aminotransferase
BMI	Body mass index
CFU	Colony forming unit
EDSS	Expanded disability status scale
ETX	Epsilon toxin
FMT	Fecal microbiota transplantation
GDH	Glutamate dehydrogenase
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HTLV	Human T-lymphotropic virus
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MBP	Myelin basic protein
MS	Multiple sclerosis
NAFLD	Non-alcoholic fatty liver disease
NAS	Non-alcoholic fatty liver disease activity score
NASH	Non-alcoholic steatohepatitis
NGS	Next-generation sequencing
PCA	Principal component analysis
PD	Potential donor

PDFF	Proton density fat fraction
PMA	Propidium monoazide
qPCR	Quantitative polymerase chain reaction
rCDI	Recurrent *Clostridioides difficile* infection
RRMS	Relapsing-remitting multiple sclerosis
SCFA	Short-chain fatty acid
SPMS	Secondary-progressive multiple sclerosis
T2DM	Type 2 diabetes mellitus
UC	Ulcerative colitis

Chapter 1

1 General Introduction

1.1 The Microbiome

1.1.1 Definitions

The two terms microbiome and microbiota are often used interchangeably in scientific research, but they have different meanings. The microbiota is defined as the microorganisms associated with a specific site on the host[1]. The microbiome was initially defined as "the ecological community of commensal, symbiotic, and pathogenic organisms that literally share our body space"[2]. The Burton Lab defines the microbiome as; the microbiota, the metagenome, and the microbial metabolites in a particular environment[3]. The metagenome is the functional potential of the microbiota[4], and the concept was first brought about in 1998 by Handelsman et al.[5] The metabolome is defined as "the complete complement of all small molecule (<1500 Da) metabolites found in a specific cell, organ or organism"[6]. It gives clues to the metabolic behaviours as it is typically representative of the end-product of metabolic actions or pathways.

1.1.2 How do we study the microbiome?

The microbiome and its components can be studied in a variety of ways. Depending on the question that needs to be addressed, a particular technique or combination of techniques can be used to assess the microbiome. Traditional methods of studying the microbiota include the use of culture-based techniques. Isolating individual bacteria from an environment and characterizing the bacteria *in vitro* can give an idea of the microbiota that constitute part of the microbiome of a given environment. This is limited by the media and conditions necessary to allow bacteria to grow. Certain bacteria have very stringent nutrient and atmospheric requirements that are difficult or currently impossible to replicate in an artificial setting and typically are provided *in vivo* by other microbial community members. It has long been estimated that approximately 1% of all bacteria on Earth are currently

culturable[7,8]. The recovery rate for bacteria originating from the human gut was approximately 20% fifteen years ago[9], but it is significantly higher in some laboratories[10]. In order to have a better understanding of the species of bacteria that are present in a sample, genomic sequencing techniques are utilized.

The microbiota can be studied using techniques that extract, amplify, and sequence the 16S ribosomal RNA (rRNA) gene from bacteria in a sample. The 16S rRNA gene is transcribed into the 16S rRNA subunit of the 30S ribosome in prokaryotes. Bacteria can be identified because the 16S rRNA gene sequence is highly conserved within a species due to the slow rate of evolution of the gene[11]. The 16S rRNA gene can be sequenced using a multitude of methods including Sanger sequencing and next-generation sequencing (NGS). Methods of NGS have evolved over time from Ion Torrent, Roche 454 system[12], to Illumina[13]. There are currently a variety of Illumina sequencers including iSeq 100 system, MiniSeq System, MiSeq Series, and NextSeq Series[14]. Each varies in the maximum number of reads per run, the run time, and maximum read length. Illumina platforms sequence short segments of DNA that can range between 150 and 300 bp[14]. The Illumina MiSeq Series has a maximum of 25 million reads per run and a maximum read length of 2 x 300 bp, which is the longest of all Illumina platforms[14]. Primers are attached to a glass chip and bridge PCR, where the DNA is fixed to a surface, is used to synthesize the complimentary strand of DNA[15]. Fluorescently labeled nucleotide bases are sequentially added one at a time to the template strand of DNA; this method is more accurate than Sanger sequencing because only one base is added at a time[16]. Lasers are used to activate the fluorescent tags and a camera detects which base has been added to millions of strands of DNA that are being sequenced at one time. The fluorescent tag is then cleaved, and the next nucleotide is added in the sequence[13]. After the Illumina run, the reads are sorted using the nucleotide sequence of barcoded primers unique for each sample; a process called demultiplexing. These reads are quality filtered to identify and correct errors made during Illumina sequencing using the software package DADA2[17]. Taxonomy is assigned for each read and a resulting table with the number of reads of each operational taxonomic unit (OTU) for each sample is

generated. Reads that have 97% similarity are grouped into an OTU, as most strains within a bacterial species have 97% similarity in their 16S rRNA gene sequence[18].

One limitation of NGS compared to culture is that an actual abundance of bacteria cannot be determined. As stated previously, Illumina® MiSeq has a fixed number of reads per run. The actual number of reads for each OTU is not a direct reflection of the total number of cells of a particular OTU within a sample. The reads for each OTU are divided by the total number of reads per sample and the relative abundance of each OTU is calculated. Despite this limitation, the benefit of using NGS is that individual bacteria do not have to be isolated and cultured from complex mixtures to be identified, which allows for identification of organisms that would otherwise be unaccounted for. However, NGS data cannot provide information on the actual abundance of bacteria.

The study of the metagenome or "the analysis of genomes contained within an environmental sample" is termed metagenomics[19]. It can be used to sequence the genes from all bacteria present in a sample. The method is especially important to identify metabolic pathways possessed by the organisms[20-22]. It can also differentiate between closely related bacteria and identify different bacterial strains within a species, as there are larger segments of DNA that are sequenced, and this can further differentiate between closely related bacteria[23-24]. Metagenomics gives a theoretical potential of the microbes in an environment, whereas metabolomic analysis provides the actual readout of what those microbes are doing at a particular point in time.

The study of these metabolic products is termed metabolomics[25]. Metabolites can be formed by the host, microbes, or originate from the environment such as food or medications[6]. The analysis is commonly performed using high-performance liquid chromatography (HPLC) and/or liquid chromatography-mass spectrometry (LC-MS)[6,26]. These techniques can identify molecules and their concentration, in both targeted and untargeted ways[26]. It must be noted that there is functional redundancy in the gut microbiome, as many microorganisms possess equivalent biochemical pathways that are able to produce the same metabolites[27]. In summary, the microbiota, metagenome and

metabolome are all important components necessary to determine microbiome composition and function.

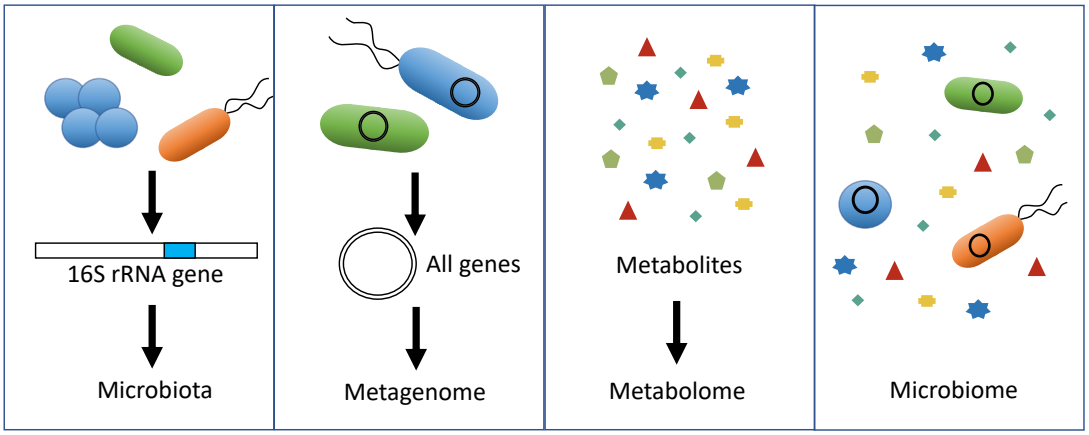

Figure 1.1 Studying the microbiota, metagenome, metabolome and microbiome.

1.1.3 The human gut microbiome

The microbiome is an important element of the human body due to the vast and complex interactions between the microbes that reside in and on the host and host physiology. It was believed that bacterial cells outnumbered human cells in the body by 10:1 [28]. This estimate was revised recently and in fact, it is more likely that the ratio of human cells to bacteria cells in/on the human body is approximately 1:1 [29]. The densest source of bacteria can be found in the human gut[30], with over 1 000 bacterial species known and every person is thought to be colonized by a minimum of 160 bacterial species[31]. The concentration of bacteria varies depending on the area of the gastrointestinal tract. In the stomach there is approximately 10^3-10^4 bacteria/mL, the duodenum and jejunum have 10^3-10^4 bacteria/mL, the ileum contains 10^8 bacteria/mL, and the colon has approximately 10^{11} bacteria/mL[29]. The increase in bacterial concentration is associated with a rise in pH, from 1.7-4.7 in the stomach, to 5.9-6.3 in the proximal small intestine, to 7.4-7.8 in the distal small intestine, to 5.0-8.0 in the colon[32].

There are a small number of bacterial genera in high abundance in the human intestine, with many more in low abundance[33]. Most human intestines are dominated by one of the three genera: *Bacteroides, Prevotella,* or *Ruminococcus*[34]. Other genera that are found in high abundance include: *Faecalibacterium*, *Eubacterium, Dorea, Alistipes,* and *Bifidobacterium*[35]. The predominant archaea detected in the human gut are methanogens, with the most abundant genera being *Methanobacterium, Methanobrevibacter,* and *Woesearchaeota*[36]. *Methanobrevibacter smithii* has been estimated to make up 10% of the anaerobic microbes found in the human colon[9].

Bacteriophage, viruses that infect bacteria and archaea, are found in the ratio of 1:10 compared to bacteria in the human gut[37]. The collection of bacteriophages, or phageome, is highly variable between individuals[38]. It has been reported that the median number of species of bacteriophage in an individual's gut is 44, and it can range between 19 to 785 [39]. The intrapersonal composition of bacteriophage remains stable over time, which indicates that bacteriophage colonize the gut[38]. The phageome may influence the gut microbiome by infecting and killing bacteria and archaea thereby altering the microbial composition[40].

The gastrointestinal tract also contains proportionally fewer fungi, collectively referred to as the mycobiome[41]. The species of fungi are quite variable between healthy individuals and change drastically over time within an individual. This indicates that these microorganisms aren't necessarily persisting in the human gut, but instead they are transient and originate from food or the oral cavity[42,43].

Fermentation of dietary fibres and protein by bacteria in the gastrointestinal tract results in the production of various compounds including short-chain fatty acids (SCFAs)[44,45]. The production of SCFAs largely takes place in the proximal colon[46]. The three most abundant SCFAs are acetate, propionate, and butyrate found in a molar ratio of approximately 60:20:20 throughout the colon[44]. Butyrate is used as a primary source of energy by colonocytes that make up the intestinal epithelia[46].

The intestinal epithelial layer is held together by tight junctions that control paracellular transport[47]. A decrease in butyrate has been hypothesized to cause an increase in intestinal permeability. Although there has been contradictory evidence from cell line models using Caco-2 cells[48,49], butyrate has been shown to increase the expression of tight junction proteins *in vitro* in Caco-2 cells[50]. Intestinal permeability is important in the context of the gut microbiome because if there is increased intestinal permeability, then microbial and dietary antigens from the intestinal lumen bypass the physical barrier that the intestine normally provides and could enter into the mucosa and circulation.

1.1.4 What causes changes in the microbiome and how can we fix them?

There are a number of factors that can influence the gut microbiome. These include method of delivery at birth[51,52], being breastfed or formula fed as an infant[51], genetics[53,54], sex[55], circadian rhythms[56], diet[57], medication use, such as antibiotics[33], as well as other drugs [58,59], and exercise[60]. Each individual has a unique gut microbiome that is like a fingerprint[31].

A healthy gut microbiome has not been defined, as there is variation in the gut microbiome between healthy people[61,62]. Microbial diversity is often used to determine if someone has a gut microbiota composition that is associated with health or disease. Diversity is calculated based on richness (the number of different species present) and evenness (how equal in abundance each species is) in a given environment[63]. The diversity of the gut microbiota is measured by alpha diversity, which is the mean species diversity of a particular community[63]. Shannon's diversity index is a measurement of alpha diversity that is used in microbiome research to compare the gut microbiota composition of multiple individuals. High diversity in the gut microbiota has been shown to be associated with health at all ages[62] and is protective against and beneficial for resolving recurrent *Clostridioides* (formerly *Clostridium*) *difficile* infection (rCDI)[64-66].

Dysbiosis is another widely used term, but its definition is a topic of debate[67]. If a healthy gut microbiome has not been defined, it is difficult to characterize its dysbiosis. Similar to the large amount of interindividual variability observed in the gut microbiome of healthy

individuals, there can also be interindividual variation in groups of people that share a common disease[68], such as inflammatory bowel disease[69]. Some research groups have attempted to classify dysbiosis into different types. Common themes include the appearance of pathogens, the loss of commensal species, or the gross change of the microbiota composition[70,71]. In theory, these different types of dysbiosis should receive different treatment therapies[67]. The appearance of pathogens could be approached with treatment methods that are specific for the pathogen that would be able to treat dysbiosis without altering other aspects of the microbiome[67]. In the case of the loss of commensal species, probiotics could be used to replenish them as long as administration was continuous as probiotic strains do not colonize[67]. If there is a gross change of the microbiota composition, fecal microbiota transplantation (FMT) may be an appropriate treatment as it can extensively change the microbiome[67].

1.2 Fecal Microbiota Transplantation

1.2.1 What is a fecal microbiota transplantation?

An FMT is the process of transplanting fecal matter from a healthy individual into the intestine of a recipient. This is not a new practice and coprophagy in humans has been documented in ancient China 1700 years ago to treat diarrhea[72,73]. In World War II it was reported that soldiers in Africa consumed camel feces as a treatment for dysentery[74]. FMT was first reported in a medical journal in 1958 to treat patients with pseudomembranous enterocolitis[75]. Since then, the practice has been greatly refined and applied to a broad range of diseases.

An FMT can be delivered in a multitude of ways including, enema, colonoscopy, endoscopy, nasoenteric tube, or capsules. Enema and colonoscopy are used to deliver the FMT to the colon. Endoscopy, nasoenteric tube or capsules are used when it would be advantageous to the deliver the fecal material to the small intestine, although these methods will also impact the colonic microbiome. The fecal sample first needs to be suspended in a fluid so that the FMT material can easily flow through syringes, tubing, or micropipettes. Water[76,77], saline[78], and/or 10% glycerol[66,79,80] are most commonly used to suspend the

fecal samples. Clinics can also vary in the equipment used to suspend the stool in liquid. Kitchen blenders were initially used to suspend the samples in clinics as they are inexpensive, but paddle blenders are now preferred as they can be used multiple times and require minimal cleaning between uses. Going forward, standardization of FMT preparation should be required to avoid substandard methods that could impact the number of viable bacteria being delivered.

1.2.2 What is being transferred?

Stool is a complex material that is comprised of water, bacteria, viruses, archaea, fungi, colonocytes, metabolites of human and microbial origin, undigested food, and inorganic compounds. It is approximately 75% water by weight with the amount of water is directly proportional to undigested fibre[81]. The typical concentration of bacteria in stool has been reported to range from 1.5 to 5 x 10^{11} bacteria/g stool, however it can differ from person to person depending on the water content of the stool[29]. There are estimated to be between 10^{8}-10^{9} virus-like particles per gram of feces, with the majority being bacteriophages[37]. Archaea are also present in stool with the most common genera being *Methanobacterium, Methanobrevibacter,* and *Woesearchaeota*[36]. Transient fungi are predominantly from the genus *Candida*[42]. As mentioned, stool also contains enterocytes that have been sloughed off from the intestinal lining, as the average intestinal epithelial cells undergoes apoptosis every four to five days[82]. Short-chain fatty acids are also present in stool, but their concentrations are decreased as a proportion are absorbed by the intestinal epithelium and further metabolized or taken up into the blood stream[44]. Healthy concentrations of SCFAs in stool are not yet known and a variety of concentrations have been reported. Acetate is found in stool at concentrations of 39.9-56.1 mM, propionate at 12.8-23.6 mM, and butyrate at 12.2-19.0 mM[83].

1.2.3 How are FMT donors selected?

As a healthy gut microbiome has yet to be defined, screening FMT donors based on their fecal microbiome is not currently practiced. Donors are selected with the assumption that if they are healthy people, their gut microbiome must be associated with health[84]. Each

donor is screened for an extensive list of infectious pathogens that may be transmitted through stool and potentially cause harm to the recipient of an FMT. Given that the practice of FMT is still recent, it is best to take a broad and cautious approach when screening donors. Therefore, there are a number of diseases that have been found to be associated with the gut microbiome and a personal and family history of these diseases are reasons for exclusion. These include but are not limited to; autoimmune diseases (multiple sclerosis[85], type 1 diabetes[86], rheumatoid arthritis[87]), obesity[88], metabolic syndrome[89], coronary disease[90], gastrointestinal disease[91-93], liver disease[94], and depression[95]. The reasoning behind screening of personal history of disease is to reduce the risk of passing along an undesired phenotype. Family members have also been shown to have similar gut microbiomes[96], and therefore family history of disease is sufficient for exclusion as the potential donor may be healthy at the time they are screened, but they could develop the disease later.

1.2.4 FMT for recurrent *Clostridioides difficile* infection

The first application of modern-day FMT was for rCDI. This Gram-positive, spore forming bacterium is the leading cause of nosocomial diarrhea. Antibiotic therapy is still the primary treatment for rCDI with vancomycin, metronidazole, and fidaxomicin being the most commonly used, even though these antibiotics may increase the risk of rCDI later on. Broad spectrum antibiotic therapy can cause drastic changes to the gut microbiota[33] and this makes patients more susceptible to recurrent infections. The chance of having a recurrence of *C. difficile* infection following one course of antibiotics has been reported to range from 10% to up to 40% [97]. If a patient has had one recurrence, the chance of having a second recurrence increases to approximately 45% [98]. The chance of having a third goes to 65% [98]. Use of antibiotics unrelated to CDI as well as being elderly, have been associated with a greater risk of recurrence[97]. An estimated 44 500 deaths occurred in the US in 2014 as a result of *C. difficile* infection[99]. There is an increased risk of mortality with recurrence[97]. Therefore, it is vital to find effective therapies for rCDI that also decrease the risk of recurrence.

The use of FMT to treat rCDI has been investigated as an alternative to antibiotics. Studies have shown that FMT is more effective than vancomycin and fidaxomicin at resolving rCDI[100-102]. One of these studies compared the effectiveness of FMT and vancomycin to vancomycin alone and found that 81% (13/16) of patients that received an FMT had resolution of rCDI versus 31% (4/13) that received only vancomycin[100]. In a clinical trial comparing the effectiveness of FMT delivered by colonoscopy versus vancomycin treatment, 90% (18/20) of FMT patients had resolution of rCDI compared to 26% (5/19) of patients that received vancomycin[101]. Likewise, another study found that FMT had the highest rate of resolution when compared to fidaxomicin or vancomycin therapy (71%, 33%, 19%, respectively)[102]. Another issue is that metronidazole and vancomycin have been associated with increased abundance of vancomycin-resistant enterococci when given for rCDI[103]. On average FMT for rCDI has a resolution rate of 92%[104]. Despite the evidence that FMT is more effective than antibiotic therapy for rCDI, it is not approved to be used as the first course of treatment yet.

The exact mechanism of action for how FMT resolves rCDI is unknown. Many believe that it is the bacteria in the suspension that are responsible for the success[105-106], by outcompeting *C. difficile* for nutrients, displacing it from its niche, and produce metabolites or signaling molecules that inhibit its growth. One hypothesis is that FMT increases the concentrations of secondary bile acids[107], produced by bacteria in the gut, as these inhibit the growth of *C. difficile*[108]. Secondary bile acids are lower in concentration in stool from rCDI patients before FMT and higher in concentration following successful FMT[108]. Stimulation of germination occurred when concentrations of primary and secondary bile acids typically found in stool from rCDI patients were incubated with clinically isolated *C. difficile* strains[108]. Germination was not induced, and vegetative growth of *C. difficile* spores was greatly reduced when concentrations of primary and secondary bile acids found in stool from rCDI patients following successful FMT were used[108].

Other studies have questioned the necessity for live bacteria being the key to resolution of rCDI. There are other components of stool that may be driving or contributing to the

success of FMT for rCDI as past studies have shown that engraftment of the donor microbiota was not necessary for resolution of rCDI, and sterile filtered FMTs, engraftment of bacteriophage, and treatment with bacterial spores have all been associated with resolution of rCDI[109-112]. Bacteriophages may limit the number of bacteria adhered to the mucosa of the intestine and therefore reduce the interactions of pathogenic bacteria with the host[113]. Due to the wide success of FMT for treating rCDI, researchers have expanded the use of FMT to a range of conditions in which the microbiome is believed to play a role.

1.2.5 FMT for other gastrointestinal diseases

Some of the most common gastrointestinal diseases that have been treated with FMT include inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis (UC), and irritable bowel syndrome (IBS). So far, these studies have had small sample sizes and varying degrees of success.

In one study, it was found that 0% (0/6) of patients with Crohn's and 25% (2/8) of patients with UC had disease remission at eight weeks post-FMT[114]. A larger study found that 77% (23/30) of Crohn's patients had disease remission one month after FMT[115], but this short follow-up is insufficient to draw conclusions. Success in a slightly longer study showed 58% (11/19) of Crohn's disease patients had improvement in symptoms twelve weeks following FMT[116]. For UC patients, a randomized controlled trial showed that 24% (9/38) of patients had remission of symptoms following FMT compared to 5% (2/37) of patients that received the placebo[117]. In another randomized control trial of UC, there was no significant difference in the efficacy of treating UC with an FMT from a healthy donor or using an autologous FMT where the patient received their own stool, 30.4% (7/23) vs 20.0% (5/25), respectively[118].

Using FMT for treatment of IBS also has a range of success reported. One meta-analysis stated that FMT for IBS was effective in 58% of patients (28/48)[119]. Halkjær et al. found that IBS patients had a greater improvement in symptoms when they were given a placebo compared to patients that received an FMT[120]. Another study treating IBS patients with

FMT that included a placebo group found that FMT was more effective at improving symptoms of IBS than the placebo, 65% (36/55) versus 43% (12/28), respectively[121].

The mechanism of action of FMT for treating other gastrointestinal diseases is not fully understood, but there have been multiple studies that have demonstrated an increase in bacterial diversity in the gut as a method of resolution. Vermeire et al.[114] found that patients who experienced remission of UC were given an FMT from donors with higher bacterial diversity than donors that did not result in remission. Vaughn et al.[116] reported that Crohn's patients that responded to FMT had an increase in fecal bacterial diversity following the FMT. Moayyedi et al.[117] found that there was an increase in bacteria diversity of UC patients that responded to FMT therapy compared to patients that received a placebo. In the case of IBS, the complexity of the condition and high efficacy of the placebo has made it difficult to understand if and how FMT is effective. Patients with IBS often have lower fecal bacterial diversity that is restored to that of healthy donors following FMT[120], but it is not clear if this is sufficient justification for the intervention. Unlike rCDI, which is caused by an infectious agent, IBS and IBD may be the result of more complex changes in the gut microbiome which are not yet fully understood. The role of the gut microbiome may be variable by patient and some may benefit from this therapy more than others.

1.2.6 FMT for distal site diseases

As the field of microbiome research has expanded, the number of conditions that have been found to have links to the microbiome has also increased. The use of FMT is now starting to be applied to these conditions that have distal site manifestations from the gastrointestinal tract. These conditions include hepatic encephalopathy[122], metabolic syndrome[123,124], multiple sclerosis (MS)[125], autism[126] and recurrent urinary tract infection[127]. These studies have low sample size numbers and firm conclusions on the effectiveness of FMT for these conditions has yet to be determined.

1.3 Non-Alcoholic Fatty Liver Disease

1.3.1 Characterization of NAFLD

Non-alcoholic fatty liver disease is an obesity-related disorder characterized by having more than 5% hepatic fat by volume and a lack of any additional cause of hepatic fat, such as alcohol consumption[128]. It affects an estimated 20-30% of North American adults and 80% of obese individuals[129]. Ten to twenty percent of NAFLD patients can progress to non-alcoholic steatohepatitis (NASH), where hepatocyte injury, inflammation, and hepatocellular ballooning are present[130]. This can sometimes be accompanied by fibrosis[131], the formation of scar tissue in the liver.

Fibrosis is not symptomatic, but it can lead to more serious complications. The stage of fibrosis is directly correlated with disease mortality[131]. Approximately 9% of NASH patients have progression of fibrosis, which can lead to cirrhosis of the liver[132]. Patients with cirrhosis can experience symptoms such as fatigue, unexplained weight loss, bruising, abdominal pain, and jaundice. Outcomes of cirrhosis can include liver failure with the potential need of a transplant, hepatocellular carcinoma, or death. In 2001, 1.2% of liver transplants were for NASH patients and that rose to 9.7% by 2009[133]. In 2013, NASH-related liver disease became the second most common indication for liver transplants in America and it is quickly becoming the leading indication for liver transplant[134]. During the first year after enlisting for a liver transplant, NASH patients have a 40.5% chance of receiving a liver and a 34.1% probability of survival while waiting for the transplant[134]. The 3-year survival rate following transplantation is 78% [133]. As NAFLD progresses, the risk of hepatocellular carcinoma increases. NASH patients are ten times more likely to develop hepatocellular carcinoma compared to NAFLD patients[132]. In North America, 12% of hepatocellular carcinoma cases are related to having NASH[135]. Hepatocellular carcinoma is present in approximately 21% of NASH patients waiting for a liver transplant in America[134].

1.3.2 Other comorbidities of NAFLD

In addition to liver damage, NAFLD patients have additional comorbidities. For example, metabolic syndrome occurs in 67% of NAFLD patients[136]. Metabolic syndrome is characterized by meeting three of more of the following criteria[137]: abdominal obesity (waist circumference >102 cm in men, > 88 cm in women); elevated plasma triglyceride levels (> 1.7 mmol/L); decreased high-density lipoprotein (HDL) cholesterol (< 1.03 mmol/L in men and < 1.30 mmol/L in women); elevated blood pressure (≥ 130/85 mmHg); or elevated fasting glucose levels (≥ 6.1 mmol/L).

Type 2 diabetes mellitus (T2DM) is another concurrent comorbidity and approximately 60% of T2DM patients have NAFLD[138]. The T2DM is a result of decreased insulin sensitivity (or insulin resistance) and increased fasting glucose levels. These patients are at an increased risk of developing neuropathy, blindness, and kidney disease from abnormally high blood glucose concentrations. The leading causes of death in NAFLD patients are cardiovascular disease related[139] due to the presence of high blood pressure, increased triglycerides and decreased HDL cholesterol. A past meta-analysis review showed that patients with NAFLD and NASH have increased intestinal permeability compared with healthy controls[140]. Miele et al.[141] discovered that NAFLD patients have significantly increased gut permeability compared to healthy controls, and the higher the gut permeability, the greater amount of fat in the liver.

1.3.3 Current treatment options for NAFLD

It is important to treat NAFLD before it progresses; at this stage of disease it is possible for fibrosis to resolve with improved lifestyle habits, such as diet and exercise. Weight loss is often recommended to NAFLD and NASH patients and it has been shown to prevent progression of fibrosis[142]. Other therapies for NAFLD include medications to better manage metabolic syndrome symptoms, which can include blood pressure medication, and metformin for the treatment of insulin resistance. While there are a number of options to treat NAFLD and prevent the progression to NASH, there is still a rising incidence in the

amount of NAFLD worldwide, likely due to the obesity epidemic. Thus, new therapies for NAFLD patients are warranted.

1.3.4 The gut microbiome and NAFLD/NASH

Many have postulated that one of the reasons that obese individuals may develop NAFLD is due to differences in the composition of bacteria in the gut compared to healthy people. There have been numerous studies that have compared the gut microbiota of NAFLD, obese, and healthy individuals and no consistent differences at the phylum or genus level have been found. One study noted that there was an increase in the relative abundance of *Lactobacillus, Dorea, Robinsoniella,* and *Roseburia* in obese NAFLD patients (n=30) compared to healthy controls (n=30)[143]. Another study reported that NASH patients (n=24) have a higher relative abundance of *Escherichia* compared to healthy controls (n=16) and obese controls without NASH (n=25)[94] , while a different study found no differences in the relative abundance of *Bifidobacterium*, Bacteroidetes, *Clostridium leptum, Clostridium coccoides, Escherichia coli* and total bacteria using qPCR when they compared the fecal microbiota composition of NAFLD patients (n=11) to healthy controls (n=27)[144]. In a study of NASH patients (n=16), they found lower relative abundances of *Faecalibacterium* and *Anaerosporobacter* and higher relative abundances of *Parabacteroides* and *Allisonella* compared to healthy controls (n=22)[145].

Additional studies reported that certain bacterial species were differentially abundant between mild/moderate NAFLD (n=72) compared to advanced fibrosis (n=14)[146]. Patients with mild/moderate NAFLD had higher relative abundances of *Ruminoccocus obeum, Eubacterium rectale,* and *Faecalibacterium prausnitzii*[146]. Patients with advanced fibrosis had higher relative abundances of *Escherichia coli* and *Bacteroides vulgatus*[146]. Boursier et al.[147] also reported that NASH patients (n=35) had a significant increase relative abundance of *Bacteroides* and a decrease in the relative abundance of *Prevotella* and advanced fibrosis patients (n=27) had a significant increase in the relative abundance of *Ruminococcus*. In a study of pediatric NAFLD, there was an association of increased *Ruminococcus* and *Dorea* with NAFLD and NASH progression[148]. Therefore, the

microbiome may play a role in developing NAFLD and progression of NAFLD to advanced fibrosis and NASH. The small samples sizes in these studies are likely the reason why the results of these microbiota studies are so variable. Future studies with larger cohort sizes are needed to determine what differences in bacterial composition exist in NAFLD and NASH patients compared to healthy controls. Then, experiments with species and strains of interest could be conducted to determine which bacteria may be contributing to or protective against the disease.

Some researchers believe that the gut microbiome of NAFLD patients has an increased energy harvesting potential, leading to weight gain. This hypothesis is based upon an obese mouse model. In 2006, Turnbaugh et al.[149] found that stool of obese mice had less energy compared to lean mice leading to the conclusion that the microbiota of obese mice could extract more energy from their diet. When fecal transplants from obese mice were given to lean mice it resulted in weight gain without changes in exercise or diet[149]. Increased energy harvesting potential of the gut microbiome has not yet been demonstrated in humans. The early work by Turnbaugh et al. has been heavily criticized in the past[150].

As opposed to certain bacteria being responsible for the pathogenesis of NAFLD, bacterial metabolites may be the driving force. It has been found that NASH patients (n=22) have significantly higher blood ethanol levels compared to healthy controls (n=16) and obese individuals (n=25)[94]. The increased ethanol could be a result of fermentation by bacteria in the gut which can lead to liver inflammation and the development of NAFLD and eventually NASH[94]. Volynets et al.[151] also found that blood ethanol levels were higher in NAFLD patients (n=20) compared to healthy controls (n=10). On the contrary, another study showed no difference in the concentration of ethanol measured from the breath of NASH patients (n=21) compared to healthy controls (n=10)[152]. Therefore, there is insufficient evidence to conclude that endogenous ethanol production by the gut microbiota is responsible for NAFLD or NASH.

As previously mentioned, NAFLD patients have an increased prevalence of abnormal intestinal permeability[140]. Several factors can contribute to increased gut permeability in

general, including genetic susceptibility[153], hyperglycemia[154], bacterial pathogens[155], and alterations to the gut microbiome. Some bacteria that make up the gut microbiota are able to ferment dietary fibre into SCFAs. As previously mentioned, butyrate increases intestinal barrier integrity, therefore if there is a decrease in butyrate production in the gut then this would lead to increased intestinal permeability. NAFLD patients may have a decrease in the abundance of butyrate producers, which causes the increased prevalence of elevated intestinal permeability.

The increase in intestinal permeability may be a driving force in the development and progression of NAFLD. It has been found that NAFLD patients have higher amounts of Gram-negative bacteria compared to healthy controls[146,156] and therefore a greater amount of lipopolysaccharide (LPS) can pass through the intestinal membrane and eventually into the portal vein due to increase intestinal permeability. LPS can cause inflammation in the liver and the chronic inflammation can contribute to the development of NAFLD and progression to NASH[157]. A decrease in butyrate and an increase in the amount of LPS passing through the intestinal membrane has been shown in mice to cause NAFLD and insulin resistance[158]. An increase in intestinal barrier integrity may reduce the amount of LPS from bacteria in the lumen of the intestine from passing through the intestinal membrane. Gao et al.[159] found that administering butyrate to mice fed a high-fat diet increased their energy expenditure and protected them from developing insulin resistance. Human studies have shown that NAFLD patients have higher levels of LPS in their blood and higher titres of antibodies to LPS[160,161].

Insulin resistance can also be associated with the microbiome as a number of studies have shown that *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* are protective against insulin resistance and their abundance is inversely related to obesity[162-164]. Hippe et al.[162] found that *F. prausnitzii* was in the highest abundance in lean healthy controls (n=18) when compared to T2DM patients (n=24) and obese controls (n=26). They noted that there were different phylotypes of *F. prausnitzii* and hypothesized that some phylotypes of *F. prausnitzii* may be more protective against T2DM than others[162]. Dao et

al.[163] found that increased *A. muciniphila* was associated with lower fasting glucose levels and waist-to-hip ratio when studying patients overweight and obese patients (n=49). Remely et al.[164] observed an increase in the abundance of *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* in T2DM patients (n=24) following weight loss. The exact mechanism for how these bacteria influence human physiology is not yet known, but many hypothesize that since the bacteria are known butyrate producers that they are beneficial for gut barrier function.

1.4 Multiple Sclerosis

1.4.1 Characterization of multiple sclerosis

Multiple Sclerosis is an autoimmune, inflammatory, demyelinating disease of the central nervous system influenced by genetic susceptibility and environmental factors[165]. Patients with MS may experience loss of motor function and vision, bladder and bowel dysfunction, muscle spasms, and cognitive impairment, which can significantly decrease their quality of life[166]. There are three main types of MS; relapsing-remitting MS (RRMS), secondary-progressive MS (SPMS), and primary-progressive MS (PPMS). The most common type of MS is RRMS, characterized by having defined relapses where there are new symptoms of MS or existing symptoms get worse, followed by complete or nearly complete recovery. There were approximately 98 000 MS cases in Canadians in 2011 and this number is expected to grow to approximately 133 000 by 2031[167]. In Canada, MS is more prevalent in females than in males[167,168]. The disease impacts life expectancy, with an estimated loss of 7 years on average[169]. Progression of the disease can be measured using the Expanded Disability Status Scale (EDSS), which ranges from 0 (healthy; no MS) to 10 (death; due to MS). The EDSS is graded using the following functional systems: pyramidal function, cerebellar functions, brain stem functions, sensory functions, visual (or optic) functions, or cerebral (or mental) functions[170].

There are no existing therapies available to stop the progression of MS. Current medications are used to slow the progression, manage relapse symptoms, and speed up recovery from relapse[165]. Steroids are used during relapses to treat inflammation in the

central nervous system, to improve recovery time and reduce the severity of symptoms[171]. Other medications, such as interferon-beta, are used to increase the time between relapses[172].

1.4.2 The gut microbiome and multiple sclerosis

Several studies have investigated the differences in the gut microbiome of MS patients compared to healthy controls. These studies have reported that MS patients do not have lower fecal microbiota diversity compared to healthy controls[173-175]. Differences between MS patients and healthy controls may take place at a lower taxonomic level as there have been reported differences in the relative abundance of specific genera and species, although the findings have varied between studies. One study reported that MS patients (n=18) had increases in the relative abundances of *Bilophila, Desulfovibrio,* and *Christensenellaceae*, and decreases in the relative abundances of *Lachnospiraceae* and *Ruminococcaceae* compared to healthy controls (n=17)[174]. A larger study reported increases of the relative abundance of *Methanobrevibacter* and *Akkermansia* and a decrease in the relative abundance of *Butyricimonas* when they compared MS patients (n=60) to healthy controls (n=43)[85]. Chen et al.[176] reported increases in the relative abundances of *Pseudomonas, Mycoplana, Haemophilus, Blautia*, and *Dorea*, and decreases in the relative abundances of *Parabacteroides, Adlercreutzia* and *Prevotella* when RRMS patients (n=31) were compared to a group of healthy controls (n=36). Cosorich et al.[177] compared nineteen RRMS patients to seventeen healthy controls and found that RRMS patients have increased abundances of *Streptococcus mitis* and *Streptococcus oralis* and a decrease in *Prevotella*. Miyake et al.[173] studied a group of Japanese RRMS patients (n=20) and compared their fecal microbiota composition to healthy controls (n=40). They found that MS patients had increases in the relative abundances of *Bifidobacterium* and *Streptococcus*, and decreases in the relative abundances of *Bacteroides, Faecalibacterium, Prevotella, Anaerostipes* and Clostridia clusters XIVa and IV[173]. They also noted that MS patients' fecal microbiota composition had more inter-individual variability compared to the group of healthy controls[173], which means that the gut microbiome of MS patients may be more difficult to characterize than originally thought. The lack of correlation between studies may be due

to experimental and analytical inconsistencies. This makes it difficult to pinpoint the key organisms that may be contributing to disease progression. It does suggest that the complex microbial community found in an FMT could be used to overcome functional changes in the microbiome rather than individual species changes.

Another potential mechanism that the gut microbiome can contribute to the development and progression of MS is the production of blood-brain barrier disrupting toxins by bacteria. *Clostridium perfringens* type B has been shown to produce the toxin "ETX" that can create holes in the blood brain barrier of MS patients[178]. One study found that 10% of RRMS patients (12/118) tested positive for ETX-specific antibodies compared to 1% of healthy controls (1/100)[178]. Molecular mimicry could also be a possible factor contributing to the development of MS, *Pseudomonas* peptides capable of activating myelin basic protein (MBP)-specific T-cell clones from MS patients[179]. A number of other bacterial species have also been proposed that produce peptides with molecular mimicry to myelin, but their prevalence in MS patients has not been studied[180].

Functionally, the most common finding is a decrease in some butyrate-producing bacteria including *Clostridium* cluster XIVa, *Faecalibacterium*, *Lachnospiraceae, Ruminococcaceae, Fusobacterium* and *Butyricimonas*[173,174,181]. A decrease in butyrate may cause an increase in intestinal permeability, allowing microbial and dietary antigens to pass through the intestinal epithelium and trigger autoimmune responses in the host. These antigens could include peptides that have molecular mimicry to myelin or bacterial toxins, thereby contributing to the development and progression of MS. Fecal microbiota transplantation could be used to deliver butyrate-producing bacteria, and these effects might be reduced[182]. More than one study noted a decrease in the relative abundance of *Prevotella*[173,176,177], a known producer of propionate, which has been shown to be correlated to increased T_H17 expansion cells and a higher prevalence of intestinal T_H17 cells[177], which are involved in the development of the disease.

The possibility that the immunomodulatory therapies used to treat MS can alter the intestinal microbiome of RRMS patients has been studied, with no differences noted in two

studies[174,176]. One study reported that MS treatment caused an increase in the relative abundances of *Prevotella* and *Sutterella*[85]. This study reported that the microbiota of MS patients currently using immunomodulatory therapy was closer in composition to healthy controls than MS patients that were treatment naïve and concluded that immunomodulatory therapy may help combat changes in the microbiome that led to the development of MS[85], but this has not been proven. The microbiome of MS patients may also play a role in relapse events. Seventeen MS patients were followed for an average of approximately twenty months and relative abundance of fusobacteria was found to be decreased in patients that had shorter lengths of time between relapses[183].

These previously mentioned studies had limited numbers of patients and their results were variable to the findings of other studies. Some observed large inter-variability between MS patients, making it difficult to define the microbiome of MS patients. Larger, multicenter studies need to take place to determine what differences exist in the gut microbiome of multiple sclerosis patients compared to healthy controls and if current therapies impact the gut microbiome. Studies using FMT are required to assess the potential to slow disease progression and improve quality of life through microbiota manipulation.

1.5 Thesis Overview

Both NAFLD and MS are important diseases worldwide due to the burden they place on the healthcare industry. While there are a number of therapies available that seek to reduce the symptoms or treat these diseases, none are particularly efficient, and the prevalence of both of these diseases are slowly increasing. As such, alternate therapies that can potentially treat these diseases or reduce the morbidity associated with them should be investigated. Given that changes to an individual's gut microbiome can potentially elicit systemic changes, FMT has the potential to be a strong candidate as a treatment or adjunct for these complicated, multifaceted diseases. This formed the basis for the enclosed thesis which comprised five objectives.

Before this could be done however, it was deemed critical to develop a standardized method for processing and storing fecal material and identifying healthy donors. Having stool banked in the clinic allows FMTs to be performed at a moment's notice. Given the difficulty of processing fecal material into a form that can be delivered and the additional difficulty of finding donors to readily donate material on short notice, determining the length of time that fecal material can be stored for was important. Little work has been carried out that investigates the length of time that fecal material can be stored while maintaining bacterial viability. This led to Objectives 1-3.

1) **Does fecal matter prepared for enema delivery stored for up to 3 months retain similar bacterial viability to fecal material that is fresh?**
2) **Does fecal matter prepared for FMT capsules stored for up to 2 months retain similar bacterial viability to fecal material that is fresh?**
3) **What proportion of healthy people qualify to be an FMT donor?**

The objectives were addressed by taking samples of healthy human feces and preparing them using existing methods for enema delivery and capsule delivery. The samples were then frozen, and they were evaluated at various time points (enema delivery: fresh, 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks; capsule delivery: fresh, encapsulated, 1 day, 3 days, 5 days, 1 week, 2 weeks, 4 weeks, 8 weeks). A maximum of 3 and 2 months were tested as most FMT clinics do not store their samples prepared for enema or capsule delivery for longer than this time frame, respectively. Once the storage time and processing methods were optimized, healthy donors were screened to determine what percentage met the inclusion criteria to be an FMT donor.

After donors were selected, the following objective was addressed:

4) **Does treating patients suffering from NAFLD with FMT from thin and healthy donors improve insulin resistance, liver proton density fat fraction, and small intestinal permeability?**

This objective was addressed by enrolling NAFLD patients in a clinical trial and measuring for a change in the primary outcome of insulin resistance, and secondary outcomes of liver proton density fat fraction (PDFF), and small intestinal permeability. A number of biochemical tests on blood were conducted to assess safety of FMT. Patients were randomized to receive an allogenic FMT from a thin and healthy donor or an autologous FMT which contained their own stool.

The final objective of this project was to determine if FMT would be a candidate therapy for treating patients that had MS.

5) **Does FMT alter the gut microbiota of RRMS patients and is this therapy safe and tolerable in RRMS patients?**

The intention was to enroll 40 patients with MS in an FMT clinical trial and measuring for changes in EDSS, small intestinal permeability and the gut microbiota composition over twelve months. However, due to the unexpected death of the principal investigator and referring clinician, this resulted in us having to complete the study with a smaller patient number (n=10). In addition to this, safety of FMT was measured in these patients. Results from this project will ultimately be utilized in order to assess if FMT for treatment of diseases beyond rCDI are able to reduce symptoms or morbidity in addition to being safe and more efficacious than current therapies available.

1.6 References

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Chapter 2

2 Commonly used methods to prepare fecal samples for fecal microbiota transplantation results in decreases in bacterial viability

2.1 Introduction

Fecal microbiota transplantation is typically used to treat rCDI. Clinics use a variety of methods to store and prepare fecal samples for transplant. Many vary in the time a sample is collected to when it is processed, the diluent used, the storage temperature, and the length of storage[1-7]. There has been a shift to the use of frozen stool after it was shown to be as effective as fresh stool in treating rCDI[1]. Some studies pre-dilute the stool samples with saline[2], water[1,3], or 10% glycerol[4-6] before long-term storage at sub-zero temperatures. Frozen stool suspended in 10% glycerol and stored for two to ten months has been used to treat rCDI, with a disease resolution rate of 88%, similar to using stool that has been stored for shorter durations[7]. While the resolution rates of rCDI has not apparently been strongly affected by the preparation methods of feces, these materials are now being expanded to treat a wide range of conditions in which the gut microbiome has been indicated. These conditions include multiple sclerosis, obesity, metabolic syndrome, irritable bowel syndrome, inflammatory bowel diseases, and cancer immunotherapeutics[8-17]. Therefore, the extent to which bacterial viability is lost with processing the fecal material needs to be minimized in case the impact on efficacy is greater in non-rCDI patients and to prevent passing along the phenotype of the previously mentioned conditions.

Traditional next-generation sequencing analysis targeting the 16S rRNA gene does not differentiate between live and dead bacteria. Propidium monoazide (PMA) is a dye that can bind covalently to DNA inside of bacterial cells with compromised cell membranes and prevent its amplification during PCR[18]. It can be used in conjunction with next-generation sequencing to sequence the DNA selectively from only the live bacteria. This method has been optimized for stool samples[19]. A past study examined the effect of oxygen exposure, freeze-thaw cycles and lag-time in processing stool samples on the relative

abundance of viable bacteria. They found that the biggest factor influencing the living profile of bacteria was the level of oxygen exposure[20]. Another study used a similar technique and found that there was a significant loss of viable bacteria and a change in composition after initial processing of stool samples for FMT[21]. A different study used PMA to examine the effect of storage time on the percentage of viable bacteria, but not the total number of viable bacteria nor the composition of viable bacteria[22].

While it has been shown that sterile fecal filtrate transfer is effective at treating rCDI[23], it is entirely possible that a decrease in bacterial viability and change in composition could impact the effectiveness of FMT for other conditions beyond rCDI. The aim of this study was to determine what storage conditions for donated stools provided the highest bacterial viability and the smallest change to the composition over time.

2.2 Methods

2.2.1 Sample Collection

Fecal samples were collected from a healthy 22-year-old female donor from the FMT clinic at St. Joseph's Health Care in London, Ontario. The donor was screened using our previously published protocol[25]. Three 80 g fecal samples were collected from the donor over a period of six months (n=3). Samples were collected at the research facility and brought directly to the lab to be processed within one hour of collection. The Western University Health Science Research Ethics Board granted ethical approval for the experiments. The experiments were carried out in accordance with the approved guidelines (REB CER 106715). Informed consent was given from the donor.

2.2.2 Sample Preparation and Storage

Whole stool samples were suspended 1:5 stool to fluid in normal sterile saline (0.9 % NaCl w/v), sterile deionized water, normal saline with 10% glycerol (v/v) or remained as whole stool. Samples were mixed inside of BA614/STR filter bags (Seward, Islandia, NY) using the Stomacher® 400 Circulator (Seward, Islandia, NY) at 230 rpm for 30 seconds. Samples were aliquoted (1 mL aliquots for liquid samples and 1 g aliquots for whole stool) and

stored in the fridge for 1 week. Time points were taken before suspension, immediately after suspension, 4 hours, 24 hours, 3 days, 5 days, and 7 days later. Samples were stored at -20 °C or -80 °C for 3 months with time points at 1 week, 2 weeks, 4 weeks, 2 months, and 3 months.

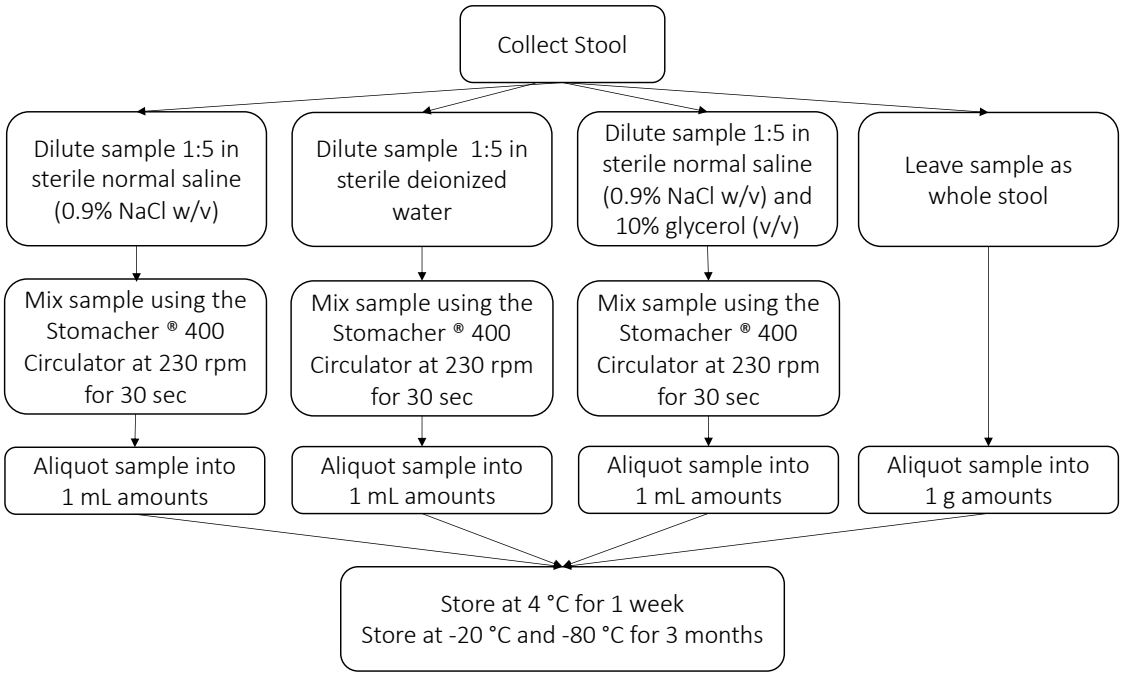

Figure 2.1 Methods for the preparation and storage of fecal samples used for FMT delivered by enema, colonoscopy, and endoscopy.

2.2.3 Bacterial Culture

At each time point the sample was serially diluted in PBS and plated on 5 different agar plates: Brain heart infusion supplemented (BHIS) agar (BD, Mississauga, ON) (selective for total anaerobes), MacConkey agar (BD) (selective for *Enterobacteriaceae*), Columbia Blood agar (BD) (selective for anaerobic staphylococci and streptococci), Columbia CNA Blood agar (BD) (selective for aerobic staphylococci, streptococci and enterococci), and Rogosa agar (BD) (semi-selective for lactobacilli and bifidobacteria). BHIS, Rogosa, and

Columbia Blood agar plates were incubated at 37 °C for 48 hours in an anaerobic chamber (10% hydrogen, 10% carbon dioxide, 80% nitrogen). Columbia CNA and MacConkey agar plates were incubated at 37 °C for 24 hours.

2.2.4 PMA Exposure

The fecal sample aliquots were diluted 1:100 in sterile PBS in an anaerobic chamber. At each time point, 2.5 µL of 20 mM PMA (Biotium, Scarborough, ON) was added to 500 µL of sample in 1.5 mL Eppendorf tubes for a final concentration of 100 µM PMA[19]. The samples were vortexed and then covered in tin foil and placed in the dark for 10 minutes. The samples were then placed lying flat on ice and exposed to light for 15 minutes (250 watts) (placed 30 cm above the samples) in a cold room. The cells were spun at 10,000 rpm for 10 minutes, the supernatant discarded and resuspended in nuclease free water three times. After the final spinning step, the sample was stored in 100 µL of nuclease free water and stored at -80 °C until time for DNA extraction.

2.2.5 DNA Extraction

DNA from the 100 µL cell pellets was extracted using the DNeasy® Powersoil® HTP 96 Kit (Qiagen, Toronto, Ontario, Canada), as per the manufacturer's instructions with the following modification: a centrifuge speed of 3700 rpm for 10 minutes was used. Extracted DNA was stored at -20 °C until amplification.

2.2.6 DNA Amplification

The BioMek® 3000 Laboratory Automation Workstation for automated PCR reagent set up was used to load 10 µL (2.3 pmol/µL) of 32 primers (16 left and 16 right) with unique barcodes into 96 well plates. Amplifications of the V4 region of the 16S ribosomal RNA gene were carried out with the primers (5′-3′) ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNxxxxxxxxGTGCCAGCMG CCGCGGTAA and (5′-3′) CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

NNNNxxxxxxxxGGACTACHVGGGTWTCTAAT (xxxxxxxx is a sample specific nucleotide barcode and the preceding sequence is a portion of the Illumina adapter sequence for library construction). The BioMek® robot was then used to transfer 2 µL of template DNA into the primer containing 96 well plates. Then 20 µl of Promega GoTaq® Colourless Master Mix (Promega, Maddison, WI) was added to the DNA template and primers. The final plate was firmly sealed with a foil PCR plate cover. This plate was placed in the Eppendorf Mastercycler® thermal cycler (Eppendorf, Mississauga, Ontario, Canada), where the lid was kept at 105 °C. An initial warm-up temperature of 95 °C was used for 2 min to activate the GoTaq®. Afterwards, the volumes underwent 25 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. After completion, the temperature of the thermal cycler was held at 4 °C, and amplicons were then stored at − 20 °C.

2.2.7 DNA Sequencing and Data Analysis

Amplified DNA was sent to the London Regional Genomics Centre at Robarts Research Institute (Western University, London, Ontario, Canada). The samples were quantified (Quant-it, Life Technologies, Burlington, Ontario, Canada) and pooled at equimolar concentrations. The pooled libraries were cleaned using QIAquick (Qiagen, Germantown, Maryland, USA) and then sequenced using the MiSeq Illumina® platform, with 2 × 300 bp paired-end chemistry. The reads were demultiplexed and filtered using dada2 (version 1.8) and custom R scripts written by Greg Gloor (github.com/ggloor/miseq_bin). The demultiplexed are available at NCBI SRA (BioProject: PRJNA557215). Taxonomy was assigned using an RDP classifier provided by the dada2 package and trained against version 132 of the SILVA database. Compositional distance between communities was quantified using the Aitchison distance with a prior count of 0.5 to avoid taking the log or ratio of zero counts and the following packages: philr, MCMCpack, tidyverse, and gtools in R[24]. Diversity of the stool microbiota was quantified based on Shannon Diversity and was calculated using the Vegan package (github.com/vegandevs/vegan) in R. Compositional distance between communities was quantified using the Aitchison distance with a prior count of 0.5 to avoid taking the log or ratio of zero counts[24]. ALDEx2 was used to identify differentially abundant taxa in R[31].

2.2.8 Statistical Analysis

Bacterial viability data at 4 °C, -20 °C and -80 °C, Aitchison distance, and Shannon diversity passed the Shapiro-Wilk test for normality. Levene's test of homogeneity was significant and as a result an ordinary one-way ANOVA with Dunnett's multiple comparison test was used to compare bacterial viability, Aitchison distance, and Shannon diversity over time within a single diluent. One-way ANOVA was used to compare bacterial viability, Aitchison distance, and Shannon diversity of different diluents at each time point with Tukey's multiple comparison test used post-hoc. Results of the one-way ANOVA tests and post-hoc tests for bacterial viability at 4 °C, -20 °C, and -80 °C can be found in Supplementary Tables 1-36. An effect size cut-off of >|3| was used with ALDEx2 to determine what bacterial taxa were differentially abundant. The Benjamini-Hochberg corrected p-value of the Wilcoxon Rank Sum test was used to identify differentially abundant taxa.

2.3 Results

2.3.1 Common methods of FMT sample preparation and storage impact bacterial survival

Changes in bacterial viability during storage at 4 °C, -20 °C and -80 °C were measured using anaerobic and aerobic culture-based techniques (Figures 2.2, 2.3, and 2.4). The samples were blended in either 10% glycerol, saline, water, or remained as whole stool. There was a significant loss of total anaerobes immediately after suspension when samples were blended in water, but not in samples that were blended with saline or 10% glycerol or remained as whole stool (Figure 2.2a). Samples stored in 10% glycerol at 4 °C did not experience a significant loss of total anaerobes throughout storage (Figure 2.2a). Samples stored in saline and as whole stool did not experience a significant loss of anaerobic bacteria until 3 days of storage (Figures 2.2a and 2.2b). Whole stool samples were the only samples to undergo a significant loss of lactobacilli throughout 7 days of storage at 4 °C (Figure 2.2c). There was no change in the concentration of bifidobacteria in samples stored in 10% glycerol or water at 4 °C for 1 week (Figure 2.2d). From 24 hours onwards of

storage at 4°C, there was no difference in the change in concentration of Gram-negative aerobes when 10% glycerol, whole stool, saline, and water were compared (Figure 2.2e). Ten percent glycerol was the only diluent to not experience a significant loss of viable Gram-positive aerobes when stored at 4 °C for 1 week (Figure 2.2f).

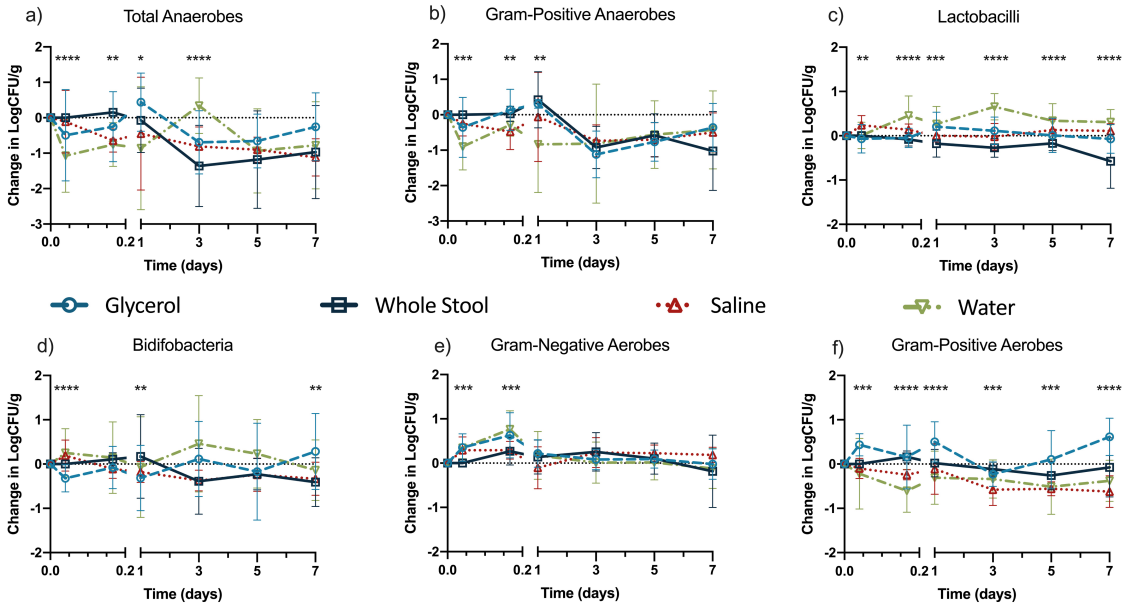

Figure 2.2 Suspension in water resulted in a significant loss of total anaerobes immediately following blending. Stool samples were suspended 1:5 in saline, water, 10% glycerol, or remained as whole stool and stored for 12 weeks. At each time point (fresh, immediately following blending, 4 hours, 1 day, 3 days, 5 days, and 7 days) a 1 mL aliquot of the sample was thawed and plated on selective media in aerobic and anaerobic conditions and incubated at 37 °C for 24-48 hours. Data displayed is three biological replicates from one donor (n=1) and each biological replicate had six technical replicates. Change in concentration was calculated by converting the CFU/mL data into logCFU/mL and subtracting the values of subsequent time points from the baseline concentration. * $p<0.05$, ** $p<0.01$, *** $p< 0.001$, **** $p<0.0001$. * on the graph are the results of the one-way ANOVA comparing diluents at each time point. One-way ANOVA was used to compare differences in the mean change in concentration of the four different diluents at each time point; Tukey's multiple comparison test was used post-hoc to compare the mean

of each diluent to each other. A detailed summary of statistics including the results of post-hoc tests can be found in Supplementary Tables 2.1-2.12.

A) Total anaerobes. After blending, 0.01 days (**p=0.0047). 4 hours, 0.167 days (**p=0.0025). 1 day (*p=0.0315). 3 days (****p<0.0001). 5 days (p=0.8215). 7 days (p=0.0850).
B) Gram-positive anaerobes. After blending, 0.01 days (***p=0.0001). 4 hours, 0.167 days (**p=0.0010). 1 day (**p=0.0018). 3 days (p=0.6730). 5 days (p=0.7733). 7 days (p=0.1132).
C) Lactobacilli. After blending, 0.01 days (**p=0.0024). 4 hours, 0.167 days (****p<0.0001). 1 day (***p=0.0002). 3 days (****p<0.0001). 5 days (****p<0.0001). 7 days (****p<0.0001).
D) Bifidobacteria. After blending, 0.01 days (****p<0.0001). 4 hours, 0.167 days (p=0.3809). 1 day (p=0.4177). 3 days (**p=0.0081). 5 days (p=0.1607). 7 days (**p=0.0651).
E) Gram-negative aerobes. After blending, 0.01 days (***p=0.0001). 4 hours, 0.167 days (***p=0.0060). 1 day (p=0.1269). 3 days (p=0.1612). 5 days (p=0.2192). 7 days(p=0.1465).
F) Gram-positive aerobes. After blending, 0.01 days (***p=0.0001). 4 hours, 0.167 days (****p<0.0001). 1 day (****p<0.0001). 3 days (***p=0.0005). 5 days (***p0.0004). 7 days (****p<0.0001).

One-way ANOVA was used to compare differences in change in concentration over time for each diluent; Dunnett's multiple comparison test was used post-hoc to compare the mean of each time point to baseline for a single diluent.

A) Total anaerobes. 10% glycerol (**p=0.0036). Whole stool (****p<0.0001). Saline (***p=0.0002). Water (**p=0.0027)
B) Gram-positive anaerobes. 10% glycerol (****p<0.0001). Whole stool (****p<0.0001). Saline (**p=0.0013). Water (p=0.1186).
C) Lactobacilli. 10% glycerol (*p=0.0481). Whole stool (****p<0.0001). Saline (**p=0.0012). Water (****p<0.0001).

D) Bifidobacteria. 10% glycerol (p=0.1383). Whole stool (**p=0.0022). Saline (****p<0.0001). Water (p=0.3703).
E) Gram-negative aerobes. 10% glycerol (****p<0.0001). Whole stool (*p=0.0204). Saline (****p<0.0001). Water (****p<0.0001).
F) Gram-positive aerobes. 10% glycerol (****p<0.0001). Whole stool (**p=0.0077). Saline (****p<0.0001). Water (*p=0.0311).

When samples were stored at -20 °C, saline had a significant decrease in the concentration of total anaerobes and Gram-positive anaerobes at 1 week of storage (Figures 2.3a and 2.3b). Samples stored in water had a significant loss of total anaerobes at 2 weeks of storage (Figure 2.3a) while samples stored in 10% glycerol or as whole stool did not experience a significant loss of total anaerobes until 3 months of storage (Figure 2.3a). Samples stored in 10% glycerol and water did not have any significant loss of lactobacilli throughout storage at -20 °C (Figure 2.3c). Samples stored suspended in saline or as whole stool had a significant loss of lactobacilli at 2 weeks' time (Figure 2.3c). Viable bifidobacteria remained stable in samples stored as whole stool (Figure 2.3d). The different diluents only differed in the concentration of viable bifidobacteria at 8 weeks of storage; whole stool was significantly higher than samples stored in 10% glycerol or water (Figure 2.3d). Samples stored in 10% glycerol were the only samples to not undergo a significant loss of Gram-negative aerobes during storage at -20 °C (Figure 2.3e). Gram-positive aerobes significantly decreased in samples suspended in saline and water at 1 week of storage (Figure 2.3f). Samples stored as whole stool did not experience any loss over the 12 weeks and samples stored in 10% glycerol did not experience a loss of Gram-positive aerobes until 12 weeks (Figure 2.3f).

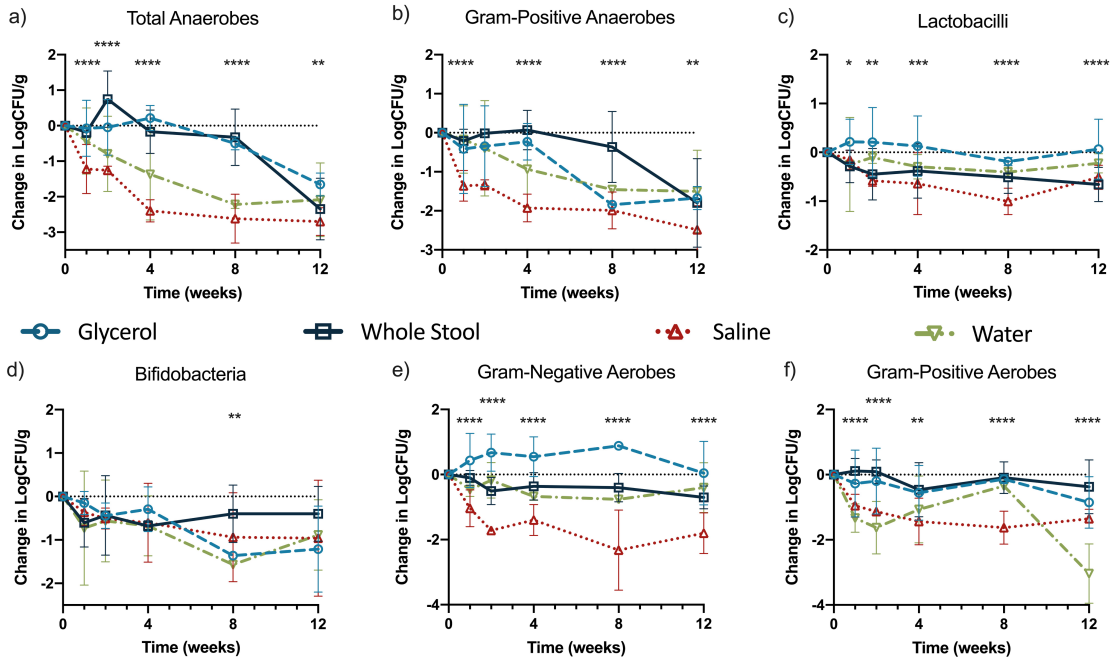

Figure 2.3 Storage as whole stool or suspended in glycerol resulted in the most stable bacterial viability at -20 °C. Stool samples were suspended 1:5 in saline, water, 10% glycerol, or remained as whole stool and stored for 12 weeks. At each time point (baseline, 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks) a 1 mL aliquot of the sample was thawed and plated on selective media in aerobic and anaerobic conditions and incubated at 37 °C for 24-48 hours. Data displayed is three biological replicates from one donor (n=1) and each biological replicate had six technical replicates. Change in concentration was calculated by converting the CFU/mL data into logCFU/mL and subtracting the values of subsequent time points from the baseline concentration. * $p<0.05$, **$p<0.01$, ***$p< 0.001$, ****$p<0.0001$. * on the graph are the results of the one-way ANOVA comparing diluents at each time point. One-way ANOVA was used to compare differences in the mean change in concentration of the four different diluents at each time point; Tukey's multiple comparison test was used post-hoc to compare the mean of each diluent to each other. A detailed summary of statistics including the results of post-hoc tests can be found in Supplementary Tables 13-24.

A) Total anaerobes. 1 week (****p<0.0001). 2 weeks (***p<0.0001). 4 weeks (***p<0.0001). 8 weeks (***p<0.0001). 12 weeks (**p=0.0011).
B) Gram-positive anaerobes. 1 week (****p<0.0001). 2 weeks (p=0.1016). 4 weeks (****p<0.0001). 8 weeks (****p<0.0001). 12 weeks (**p=0.0034).
C) Lactobacilli. 1 week (*p=0.0388). 2 weeks (**p=0.0011). 4 weeks (***p=0.0008). 8 weeks (****p<0.0001). 12 weeks (****p<0.0001).
D) Bifidobacteria. 1 week (p=0.1249). 2 weeks (p=0.9620). 4 weeks (p=0.3414). 8 weeks (**p=0.0049). 12 weeks (p=0.1594).
E) Gram-negative aerobes. 1 week (****p<0.0001). 2 weeks (****p<0.0001). 4 weeks (****p<0.0001). 8 weeks (****p<0.0001). 12 weeks (****p<0.0001).
F) Gram-positive aerobes. 1 week (****p<0.0001). 2 weeks (****p<0.0001). 4 weeks (**p=0.0037). 8 weeks (****p<0.0001). 12 weeks (****p<0.0001).

One-way ANOVA was used to compare differences in change in concentration over time for each diluent; Dunnett's multiple comparison test was used post-hoc to compare the mean of each time point to baseline for a single diluent.

A) Total anaerobes. 10% glycerol (****p<0.0001). Whole stool (****p<0.0001). Saline (****p<0.0001). Water (****p<0.0001).
B) Gram-positive anaerobes. 10% glycerol (****p<0.0001). Whole stool (****p<0.0001). Saline (****p<0.0001). Water (****p<0.0001).
C) Lactobacilli. 10% glycerol (p=0.5214). Whole stool (****p<0.0001). Saline (****p<0.0001). Water (p=0.3037).
D) Bifidobacteria. 10% glycerol (****p<0.0001). Whole stool (p=0.0624). Saline (**p=0.0082). Water (**p=0.0028).
E) Gram-negative aerobes. 10% glycerol (**p=0.0044). Whole stool (****p<0.0001). Saline (****p<0.0001). Water (**p=0.0016).
F) Gram-positive aerobes. 10% glycerol (*p=0.0301). Whole stool (**p=0.0074). Saline (****p<0.0001). Water (****p<0.0001).

When samples were stored at -80 °C, samples stored as whole stool did not experience any significant loss of total anaerobes or Gram-positive anaerobes throughout 3 months of

storage (Figure 2.4a and b). Samples stored in 10% glycerol, saline, and water had significant losses of total anaerobes at 1 week of storage at -80 °C (Figure 2.4a). Samples stored in 10% glycerol and water did not have any significant changes in the concentration of lactobacilli throughout storage (Figure 2.4c). Samples stored as whole stool did not have any significant decreases in the concentration of bifidobacteria over time (Figure 2.4d). Only samples stored in saline had a loss of Gram-negative aerobes throughout storage and this started at 1 weeks' time (Figure 2.4e). Samples stored in 10% glycerol and whole stool did not have a significant loss of Gram-positive aerobes (Figure 2.4f).

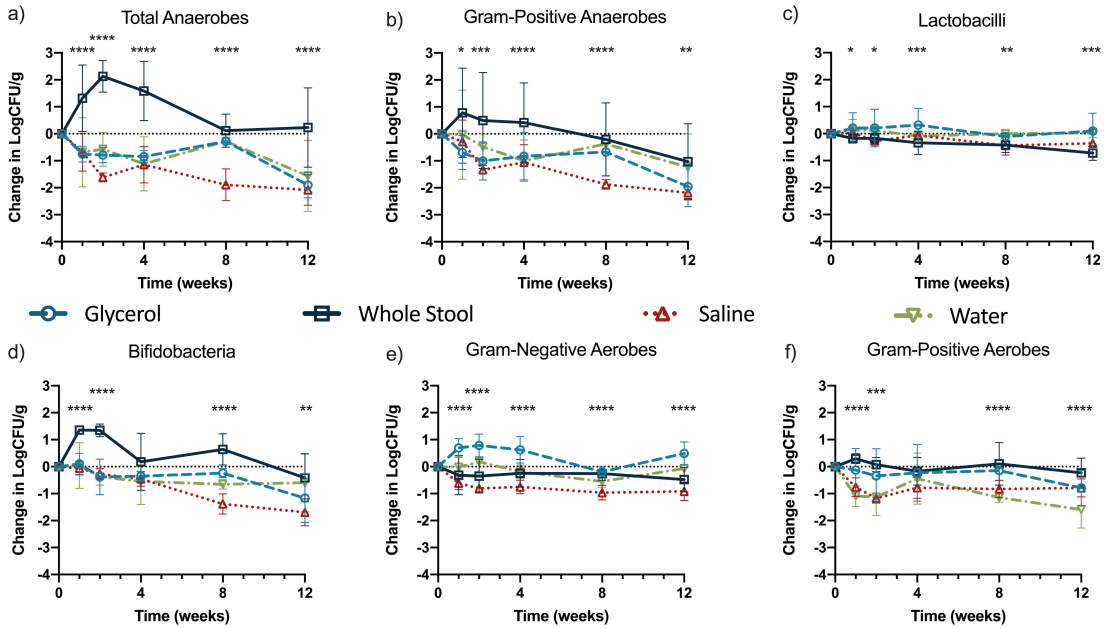

Figure 2.4 Storage as whole stool resulted in the most stable bacterial viability at -80°C. Stool samples were suspended 1:5 in saline, water, 10% glycerol, or remained as whole stool and stored for 12 weeks. At each time point (baseline, 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks) a 1 mL aliquot of the sample was thawed and plated on selective media in aerobic and anaerobic conditions and incubated at 37 °C for 24-48 hours. Data displayed are three biological replicates from one donor (n=1) and each biological replicate had six technical replicates. Change in concentration was calculated by converting the CFU/mL data into logCFU/mL and subtracting the values of subsequent time points

from the baseline concentration. * p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001. * on the graph are the results of the one-way ANOVA comparing diluents at each time point. One-way ANOVA was used to compare differences in the mean change in concentration of the four different diluents at each time point; Tukey's multiple comparison test was used post-hoc to compare the mean of each diluent to each other. A detailed summary of statistics including the results of post-hoc tests can be found in Supplementary Tables 25-36.

A) Total anaerobes. 1 week (****p<0.0001). 2 weeks (****p<0.0001). 4 weeks (****p<0.0001). 8 weeks (****p<0.0001). 12 weeks (****p<0.0001).
B) Gram-positive anaerobes. 1 week (*p=0.0131). 2 weeks (***p=0.0003). 4 weeks (****p<0.0001). 8 weeks (****p<0.0001). 12 weeks (**p=0.0028).
C) Lactobacilli. 1 week (*p=0.0480). 2 weeks (*p=0.0247). 4 weeks (***p=0.0001). 8 weeks (**p=0.0029). 12 weeks (***p=0.0001).
D) Bifidobacteria. 1 week (****p<0.0001). 2 weeks (****p<0.0001). 4 weeks (p=0.0549). 8 weeks (****p<0.0001). 12 weeks (**p=0.0028).
E) Gram-negative aerobes. 1 week (****p<0.0001). 2 weeks (****p<0.0001). 4 weeks (****p<0.0001). 8 weeks (****p<0.0001). 12 weeks (****p<0.0001).
F) Gram-positive aerobes. 1 week (****p<0.0001). 2 weeks (***p=0.0006). 4 weeks (p=0.0876). 8 weeks (****p<0.0001). 12 weeks (****p<0.0001).

One-way ANOVA was used to compare differences in change in concentration over time for each diluent; Dunnett's multiple comparison test was used post-hoc to compare the mean of each time point to baseline for a single diluent.

A) Total anaerobes. 10% glycerol (****p<0.0001). Whole stool (****p<0.0001). Saline (****p<0.0001). Water (****p<0.0001).
B) Gram-positive anaerobes. 10% glycerol (****p<0.0001). Whole stool (**p=0.0067). Saline (****p<0.0001). Water (**p=0.0042).
C) Lactobacilli. 10% glycerol (p=0.4776). Whole stool (****p<0.0001). Saline (****p<0.0001). Water (p=0.0622).

D) Bifidobacteria. 10% glycerol (****p<0.0001). Whole stool (****p<0.0001). Saline (****p<0.0001). Water (*p=0.0276).
E) Gram-negative aerobes. 10% glycerol (****p<0.0001). Whole stool (p=0.1095). Saline (****p<0.0001). Water (**p=0.0100).
F) Gram-positive aerobes. 10% glycerol (p=0.0570). Whole stool (*p=0.0158). Saline (****p<0.0001). Water (****p<0.0001).

2.3.2 The composition of viable bacteria is stable during storage

Differences between baseline microbiota composition and subsequent time-points were quantified using the Aitchison distance which provides a compositionally robust alternative to standard β-diversity metrics[24]. Aitchison distance increases as the bacterial composition of two samples are less similar to one another. There was a significant initial increase in Aitchison distance following collection of the stool sample (Figure 2.5a). Aitchison distance was stable throughout the duration of storage at 4 °C, -20 °C, and -80 °C for samples stored in 10% glycerol, whole stool saline, and water (Figure 2.5). The resulting Aitchison distance of different diluents was compared at each time point for storage at 4 °C, -20 °C, and -80°C. Diluents did not differ in Aitchison distance when stored at 4 °C (Figure 2.5a). When stored at -20 °C, water had significantly lower Aitchison distances compared to whole stool and saline at one week of storage (Figure 2.5b). There were no differences in Aitchison distance at other time points. When stored at -80 °C, water had significantly lower Aitchison distances compared to whole stool and saline when stored for 1 month and 3 months. When samples were stored for 2 months at -80 °C, 10% glycerol had significantly lower Aitchison distances compared to whole stool and saline (Figure 2.5c). These findings indicated that there was no superior diluent when samples were stored at 4 °C and -20 °C, but when samples were stored at -80 °C, whole stool and saline experienced the largest change in the composition of viable bacteria.

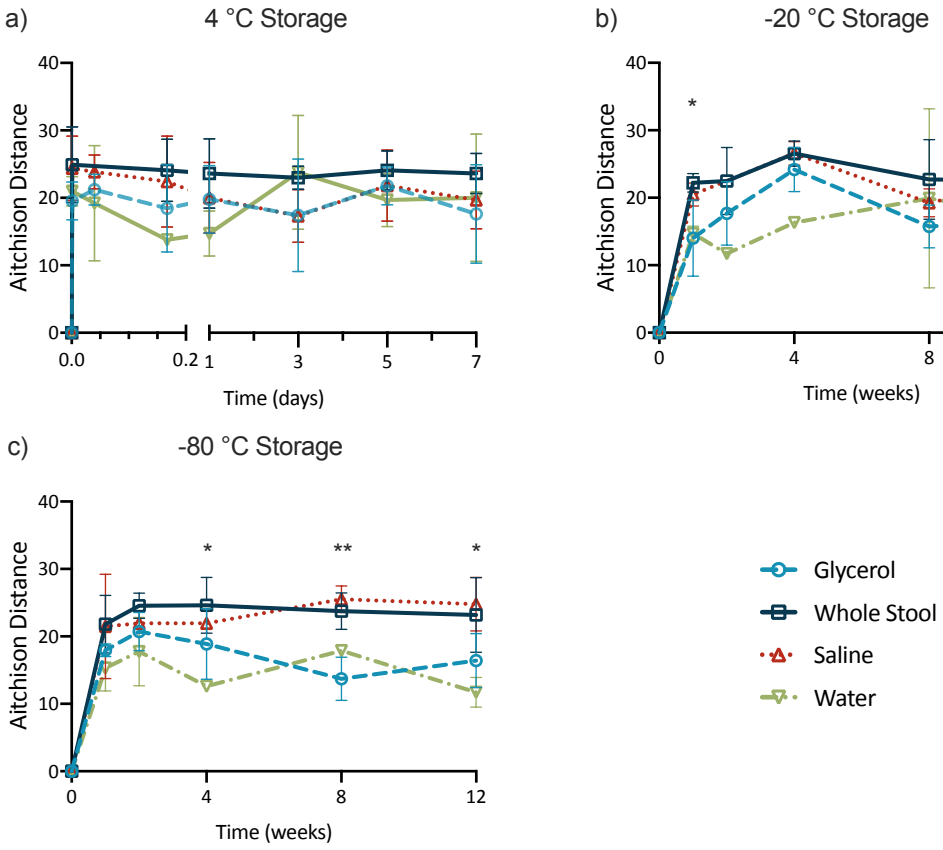

Figure 2.5 The greatest change in the composition of viable bacteria occurs after initial sample collection. Stool samples were suspended 1:5 in saline, water, 10% glycerol, or remained as whole stool and stored for one week at 4 °C or twelve weeks at -20 °C and -80 °C. At each time point (fresh: baseline, immediately following preparation, 4 hours, 1 day, 3 days, 5 days, 7 days; frozen: baseline, 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks) a 1 mL aliquot of the sample was thawed and treated with PMA under anaerobic conditions and sent for next-generations sequencing. Aitchison distance was calculated on the resulting sequencing reads. Data displayed are three biological replicates from one donor (n=1). * $p<0.05$, **$p<0.01$, ***$p< 0.001$, ****$p<0.0001$. * on the graph are the results of the one-way ANOVA comparing diluents at each time point. One-way ANOVA was used to compare differences in the mean Aitchison distance of the four different diluents at each time point; Tukey's multiple comparison test was used post-hoc to compare the mean of each diluent to each other.

A) Samples stored at 4 °C. Fresh, 0.001 days (p=0.3649). After blending, 0.01 days (p=0.5938). 4 hours, 0.167 days (p=0.1478). 1 day (p=0.2270). 3 days (p=0.4727). 5 days (p=0.6080). 7 days (p=0.8217)
B) Samples stored at -20 °C. 1 week (*p=0.0440; post-hoc whole stool vs water: **p=0.0018). 2 weeks (p=0.1028). 4 weeks (p=0.1055). 8 weeks (p=0.6716). 12 weeks (p=0.1515).
C) Samples stored at -80 °C. 1 week (p=0.2883). 2 weeks (p=0.1688). 4 weeks (*p=0.0233; post-hoc whole stool vs water: **p=0.0076; post-hoc saline vs water: ***p=0.0004). 8 weeks (**p=0.0065; post-hoc 10% glycerol vs whole stool: *p=0.0145; post-hoc 10% glycerol vs saline: **p=0.0056). 12 weeks (*p=0.0149; post-hoc whole stool vs water: *p=0.0289; post-hoc saline vs water: **p=0.0075).

One-way ANOVA was used to compare differences in change in Aitchison distance over time for each diluent; Dunnett's multiple comparison test was used post-hoc to compare the mean of each time point to baseline for a single diluent.

A) Samples stored at 4 °C. 10% glycerol (p=0.9346). Whole stool (p=0.9956). Saline (p=0.5476). Water (p=0.4788)
B) Samples stored at -20 °C. 10% glycerol (p=0.0765). Whole stool (p=0.8198). Saline (p=0.4057). Water (p=0.6790).
C) Samples stored at -80 °C. 10% glycerol (p=0.2335). Whole stool (p=0.8955). Saline (p=0.6661). Water (p=0.1977).

The largest change in composition occurred during the initial collection of stool samples from the donor (Figure 2.5). The five most abundant genera in the fresh fecal samples were *Bacteroides*, *Faecalibacterium*, *Alistipes*, *Parabacteroides*, and *Parasutterella*. ALDEx2 was used to compare the composition of fresh fecal samples with and without PMA that had not been processed for FMT; this comparison is an indication of the impact the collection process in aerobic conditions had on viable bacterial composition. An effect size cut-off of >|3| was used. There were no bacterial taxa that had an effect size of >|3|.The Benjamini-Hochberg corrected p-value of the Wilcoxon Rank Sum test was used to identify differentially abundant taxa in order to compare the results of this study to findings

from previously published studies that did not use effect size to identify differentially abundant taxa[20-22]. It was found that the relative abundances of *Alistipes, Bacteroides,* and *Parasutterella* increased when the stool samples were exposed to oxygen during collection (Figure 2.6). Three *Alistipes* OTUs increased in relative abundance during sample collection (*Alistipes* OTU 1: effect size= 1.305, p-value= 0.0079; *Alistipes* OTU 2: effect size= 1.141, p-value= 0.0011; *Alistipes* OTU 3: effect size= 1.161, p-value= 0.00096). Six *Bacteroides* OTUs increased in relative abundance during sample collection. (*Bacteroides* OTU 1: effect size=1.063, p-value=0.018; *Bacteroides* OTU 2: effect size= 1.493, p-value=0.0057; *Bacteroides* OTU 3: effect size=1.092, p-value=0.015; *Bacteroides* OTU 4: effect size=1.195, p-value=0.018; *Bacteroides* OTU 5: effect size=1.449, p-value=0.0048; *Bacteroides* OTU 6: effect size=1.141, p-value=0.016). One *Parasutterella* OTU increased in relative abundance during sample collection (effect size=1.100, p-value=0.028).

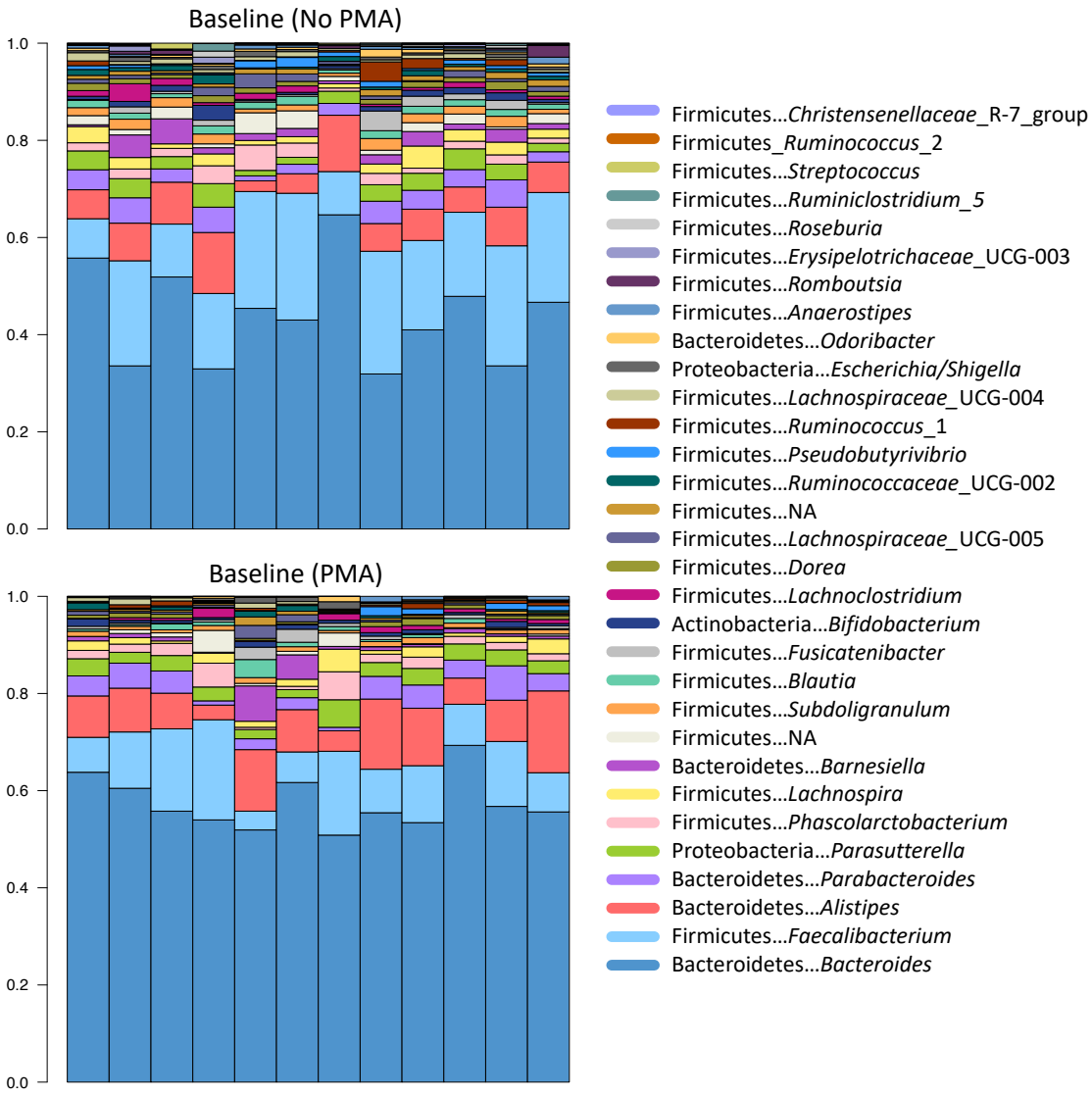

Figure 2.6 Significant changes in the composition of viable bacteria occur during initial collection of stool samples. Fresh stool samples were collected from one healthy donor at the research facility and processed with PMA within one hour of collection. The samples presented are from baseline, before any diluent or storage had taken place. They represent the viable bacteria in stool within the body (Baseline No PMA) and the viable bacteria in stool after exposure to oxygen during the collection process (Baseline PMA). DNA from each sample was extracted and sent for next-generation sequencing. Each column represents one biological replicate from the donor (n=1). The R package ALDEx2

was used to calculate effect size to determine significant changes in viable relative abundance of bacteria after stool sample collection. Three *Alistipes* OTUs increased in relative abundance during sample collection (*Alistipes* OTU 1: effect size= 1.305, p-value= 0.0079; *Alistipes* OTU 2: effect size= 1.141, p-value= 0.0011; *Alistipes* OTU 3: effect size= 1.161, p-value= 0.00096). Six *Bacteroides* OTUs increased in relative abundance during sample collection. (*Bacteroides* OTU 1: effect size=1.063, p-value=0.018; *Bacteroides* OTU 2: effect size= 1.493, p-value=0.0057; *Bacteroides* OTU 3: effect size=1.092, p-value=0.015; *Bacteroides* OTU 4: effect size=1.195, p-value=0.018; *Bacteroides* OTU 5: effect size=1.449, p-value=0.0048; *Bacteroides* OTU 6: effect size=1.141, p-value=0.016). One *Parasutterella* OTU increased in relative abundance during sample collection (effect size=1.100, p-value=0.028).

2.3.3 Diversity of viable bacteria remains stable throughout the storage process.

Shannon diversity was calculated and bacterial diversity over time within a diluent and between diluents were compared. There was not a significant change in diversity of samples stored in 10% glycerol, whole stool, saline, or water over time at 4 °C, -20 °C or -80 °C (Figure 2.7a, b, c). When stored at -20 °C at two weeks' time, water had significantly lower viable bacterial diversity than 10% glycerol or whole stool (Figure 2.7b). There were no time points that the diversity of viable bacteria differed between the diluents or whole stool when stored at -80 °C (Figure 2.7c).

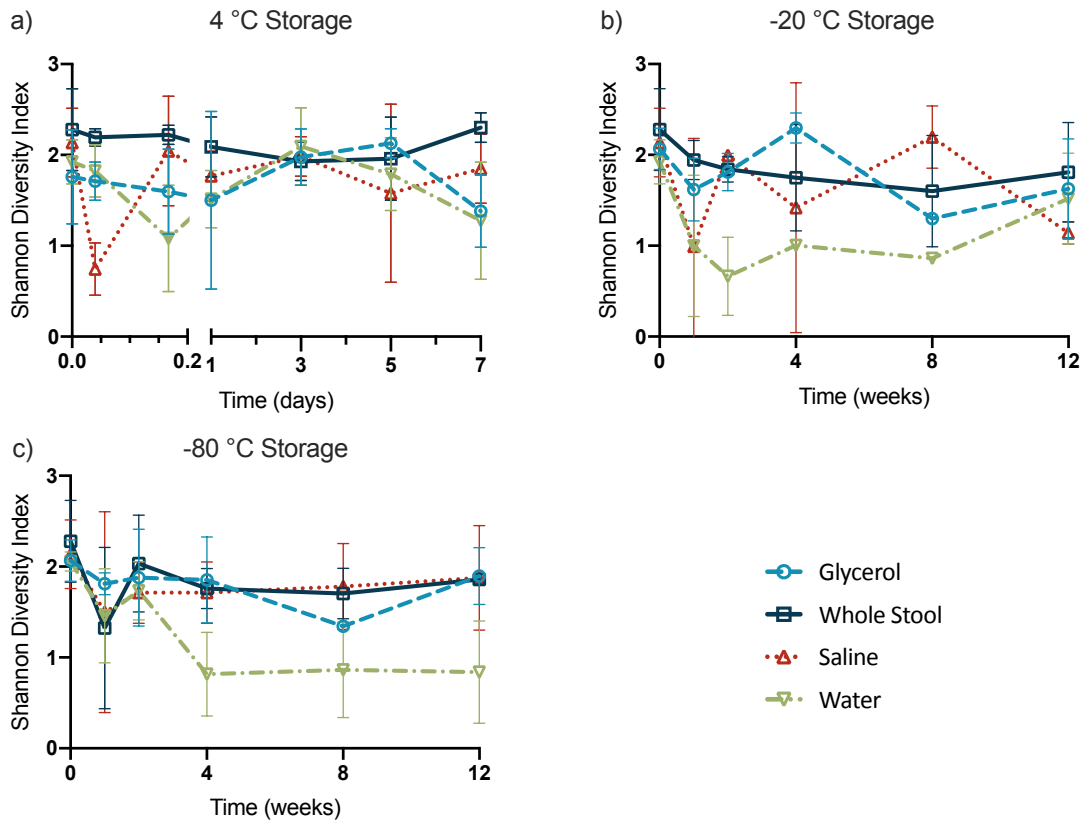

Figure 2.7 Duration of storage did not significantly decrease the diversity of viable bacteria. Stool samples were suspended 1:5 in saline, water, 10% glycerol, or remained as whole stool and stored for one week at 4 °C or twelve weeks at -20 °C and -80 °C. At each time point (fresh: baseline, immediately following preparation, 4 hours, 24 hours, 3 days, 5 days, 7 days; frozen: baseline, 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks) a 1 mL aliquot of the sample was thawed and treated with PMA under anaerobic conditions and sent for next-generations sequencing. Shannon diversity index was calculated on the resulting sequencing reads. Data displayed are three biological replicates from one donor (n=1). * $p<0.05$, ** $p<0.01$, *** $p< 0.001$, **** $p<0.0001$. * on the graph are the results of the one-way ANOVA comparing diluents at each time point. One-way ANOVA was used to compare differences in the mean Shannon diversity index of the four different diluents at each time point; Tukey's multiple comparison test was used post-hoc to compare the mean of each diluent to each other.

A) Samples stored at 4 °C. Fresh, 0.001 days (**p=0.0088; whole stool vs water: ***p=0.0008; saline vs water: *p=0.0291). After blending, 0.01 days (**p=0.0098; 10% glycerol vs whole stool: *p=0.0352; 10% glycerol vs saline: *p=0.0282; whole stool vs saline: **p=0.0012; saline vs water: *p=0.0102). 4 hours, 0.167 days (p=0.0802). 1 day (p=0.4580). 3 days (p=0.9210). 5 days (p=0.7002). 7 days (p=0.1339)
B) Samples stored at -20 °C. 1 week (p=0.3146). 2 weeks (*p=0.0102; 10% glycerol vs water: *p=0.0247; whole stool vs water: *p=0.0182). 4 weeks (p=0.4898). 8 weeks (p=0.2309). 12 weeks (p=0.4033).
C) Samples stored at -80 °C. 1 week (p=0.8478). 2 weeks (p=0.8294). 4 weeks (p=0.1048). 8 weeks (p=0.1977). 12 weeks (p=0.1196).

One-way ANOVA was used to compare differences in change in Shannon diversity index over time for each diluent; Dunnett's multiple comparison test was used post-hoc to compare the mean of each time point to baseline for a single diluent.

A) Samples stored at 4 °C. 10% glycerol (p=0.5694). Whole stool (p=0.0531). Saline (p=0.0686). Water (p=0.6896)
B) Samples stored at -20 °C. 10% glycerol (p=0.1023). Whole stool (p=0.5948). Saline (p=0.3345). Water (p=0.0.2564).
C) Samples stored at -80 °C. 10% glycerol (p=0.6993). Whole stool (p=0.4506). Saline (p=0.8268). Water (p=0.1097).

2.4 Discussion

This study showed that some preparatory and storage methods used by FMT clinics result in loss in the number and change in composition of viable bacteria. This may impact the success and long-term outcomes of FMT. The is the first description of using PMA in conjunction with next-generation sequencing to investigate storage duration and diluents (water, saline, 10 % glycerol) on the composition of viable bacteria in feces during long-term storage and it provided data that should be useful to people performing FMT in the future.

There was a significant initial loss of viable bacteria when samples were blended with water (Figure 2.2) and this effect was not observed in samples blended in saline or 10% glycerol. This was likely due to the hypotonic nature of water that may have caused bacterial cell lysis. The diluent used was significant in storage at -20 °C (Figure 2.3) and -80 °C (Figure 2.4). These findings suggest that the effect of freeze-thawing different diluents could be a contributing factor to bacterial loss during long-term storage. Storage in saline and water resulted in greater losses of viable bacteria than storage in whole stool or 10% glycerol when stored at -20 °C and -80 °C. Ten percent glycerol is a known cryoprotectant and whole stool may behave in the same way due to its limited water content. The cell death observed in samples stored in saline and water could be caused by greater amounts of ice crystals formed during the freeze-thaw process that sheared the bacterial cells. Samples stored at -80 °C (Figure 2.4) experienced less total bacterial loss compared to the same samples stored at -20 °C (Figure 2.3). Many FMT clinics do not have access to a -80 °C freezer, but it could help to preserve the donor stool for longer. Screening donors for FMT can be costly[25]. Being able to store stool for longer durations will allow clinics to bank higher quantities of stool from donors between their rescreening visits, and potentially reduce the frequency that donors will have to be rescreened.

The largest change in Aitchison distance, and therefore the greatest change in the composition of viable bacteria, occurred after the initial collection of the stool sample, without any further processing (Figure 2.5a). Each preparation method resulted in a similar initial increase in Aitchison distance, this was likely a result of oxygen exposure. These samples were collected at the lab site and were processed within an hour of collection. In normal practice, samples are typically delivered within four hours of collection and processed when they arrive at the clinic, so it is possible that this change in composition could be greater in these centres. After the initial increase in Aitchison distance, the composition of the viable bacteria in stool remained stable at 4 °C for one week and -20 °C and -80 °C for three months within each diluent (Figure 2.5). For samples stored at -80 °C, whole stool and saline resulted in significantly higher Aitchison distances at 1 month, 2 months, and 3 months of storage (Figure 2.5c). Storage in 10% glycerol and water may

cause proportional death of microbes, which is why they resulted in a lower Aitchison distance as the composition of viable microbes stayed more similar to baseline.

We found that the relative abundances of *Alistipes, Bacteroides* and *Parasutterella* increased following stool collection (Figure 2.6). This does not mean that these bacteria replicated during storage, but rather the treatment with PMA and the compositional nature of this data caused bacteria that died to decrease in relative abundance and made these genera appear to increase in relative abundance. Other researchers have found similar losses during the initial sample collection period. One study noted that the genera that decreased after oxygen exposure were *Faecalibacterium, Subdoligranulum, Eubacterium hallii, Eubacterium rectale, Roseburia* and *Anaerostipes*[21]. They found that *Escherichia/Shigella* and *Alistipes* increased after oxygen exposure[21]. All the previous studies examining the composition of stool for FMT have found a decrease in the relative abundance of *Faecalibacterium* following exposure to oxygen during sample collection[20-22]. Another study that utilized PMA to investigate fecal sample collection for FMT also noted that there was an increase in the relative abundance of *Alistipes*, indicating that these bacteria are able to survive these conditions[21]. Potentially, methods to collect stool samples anaerobically should be implemented by FMT clinics and stool banks to reduce the change in viable composition that occurs during the collection phase. One study suggested collecting samples in a Stomacher® 400 bag inside of an anaerobic jar and processing the sample under anaerobic conditions to overcome this problem[22].

Previous studies have investigated the effect of different diluents on the survival of bacteria for FMT including: normal saline, saline solutions with maltodextrin and trehalose[22], 10% glycerol solutions[21], 50% glycerol solutions and the use of cysteine buffers[20], but none have directly compared the suspension fluids currently used by FMT clinics. We found that not all diluents had the same effect on viable bacterial diversity when stored at 4 °C or -20°C (Figure 2.7). Diversity remained stable over time when samples were stored at -80 °C diluted in 10% glycerol, saline, water or as whole stool (Figure 2.7c), however water resulted in the lowest diversity. These findings suggest that certain diluents, or lack thereof,

are superior for long-term storage of stool samples for FMT. An increased diversity in donor stool has been shown to be highly beneficial when treating rCDI[26] as well as inflammatory bowel diseases[27,28]. Diluting samples in water before long-term storage resulted in the largest decrease in diversity over time and this method of preparation and storage is not recommended for FMT stool processing. Suspension in water resulted in an immediate reduction in the concentration of viable total anaerobes as well (Figure 2.2a).

The uniqueness of this study was the use of PMA-seq to determine changes in the composition of viable bacteria in stool samples, and the data showing that commonly used diluents affect the composition of viable bacteria. A limitation of this study was that only one donor was used to measure changes over time. Past studies have shown that different donor stools undergo varying changes in viable bacterial composition and concentration[21,22]. There can be significant variations in the composition of the gut microbiota between individuals, so even if more than one donor had been sampled, comparisons could only be made within not between each individual. It is possible that baseline PMA treated samples had an altered composition because not all bacteria in stool are viable when passed; some may have died within the body before being exposed to oxygen. Not all bacteria are culturable and while a variety of selective media was used to recover bacteria, some bacteria may not have been cultured, and the total number of viable organisms may have been underestimated.

Successful FMT for rCDI has been reported using sterile fecal transplants[23], spores[29], and donor bacteriophage[30] so the need for viable bacteria may not be paramount. However, until the mechanisms of action of FMT are understood for the different potential applications, fecal samples should be stored in ways that maintain the viability of as many bacteria as possible. Optimization of the preparation and storage conditions of FMT at -80 °C in 10% glycerol or whole stool has the potential to improve efficacy and is recommended for FMT clinics.

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Chapter 3

3 Characterization of viable bacteria throughout preparation and storage of fecal microbiota transplantation capsules

3.1 Introduction

Fecal microbiota transplantation has proven to be an effective method for treating recurrent rCDI. Fresh stool was first used to treat rCDI with FMT[1-5] until frozen stool was found to be as effective and a shift began to use frozen material for FMT[6-8]. These FMT studies used a variety of delivery methods including: colonoscopy[2,3,5,7], enema[4,8], nasogastric[1], or nasojejunal tube[4] and there were no differences in success of curing rCDI. While FMT is an effective method for treating rCDI, there is still a stigma surrounding the procedure, as some patients believe it is unpleasant or unappealing.

FMT capsules are emerging as a favoured alternative to the previously mentioned methods as they are better tolerated, less invasive and more cost-effective[9]. Capsules have been shown to be just as effective as FMT delivered via other methods. Kao et al.[9] showed that capsules had a resolution rate of 96% in both their capsule and colonoscopy treated groups. This result is comparable to Hirsch et al.[10] that found an 89% resolution rate when treating patients with FMT capsules and Youngster et al.[11,12] that found resolution rates of 90-91% after 2 courses of FMT capsules.

The majority of bacteria in stool are anaerobic and there is an increased processing time to manufacture FMT capsules versus an enema or colonoscopy delivered FMT. While the resolution rates of capsules are comparable to other FMT methods, this new processing technique may impact the number and composition of viable bacteria present in the capsules. FMT therapy is being tested for an increasing list of medical conditions, such as: metabolic syndrome[13,14], inflammatory bowel diseases[15-17], and irritable bowel syndrome [18,19]. Changes to the composition of viable bacteria in an FMT may impact the effectiveness for treating other diseases. Past studies have documented changes in viable bacteria when fecal samples were prepared for colonoscopy or enema procedures[20-22], but no one has

investigated if the process of manufacturing FMT capsules influences the composition of viable bacteria. The aim of this study was to determine if the encapsulation of fecal material resulted in changes to the number and composition of viable bacteria delivered. Culture-based and 16S rRNA gene sequencing analyses were conducted to answer this question. Propidium monoazide was used in conjunction with 16S rRNA gene analysis to sequence only live bacteria.

3.2 Methods

3.2.1 Sample Collection

Fecal samples were collected from three healthy donors from a local FMT clinic, one male and two females with a median age of 22 (range 21-24 years old). Donors were screened using a previously published protocol[23]. The experiments were carried out in accordance with the approved guidelines (REB CER 106715). Informed consent was given from the donors. The encapsulation process was started within one hour of sample collection.

3.2.2 FMT Capsule Preparation

Eighty to one hundred grams of donor stool was mixed with 200 mL of sterile normal saline (0.9%) and 40 mL of 100% glycerol inside of a BA614/STR filter bag (Seward, Islandia, NY) using the Stomacher® 400 Circulator (Seward, Islandia, NY) at 230 rpm for 30 seconds. Following this step, the slurry was aliquoted into 15 mL conical tubes and centrifuged at 400 g for 20 minutes at 4 °C to remove the remaining fibre. The supernatant was collected and centrifuged at 10 000 *x* g for 30 minutes at 4 °C. The supernatant was discarded and 450 μL of the resulting pellet was used to fill each No. 1 gelatin capsules (Medisca, Plattsburgh, NY), which were then encapsulated with No. 0 (Medisca, Plattsburgh, NY) and No. 00 capsules (Medisca, Plattsburgh, NY). A single donor sample produced approximately 40 capsules and they were stored at -80°C for 2 months.

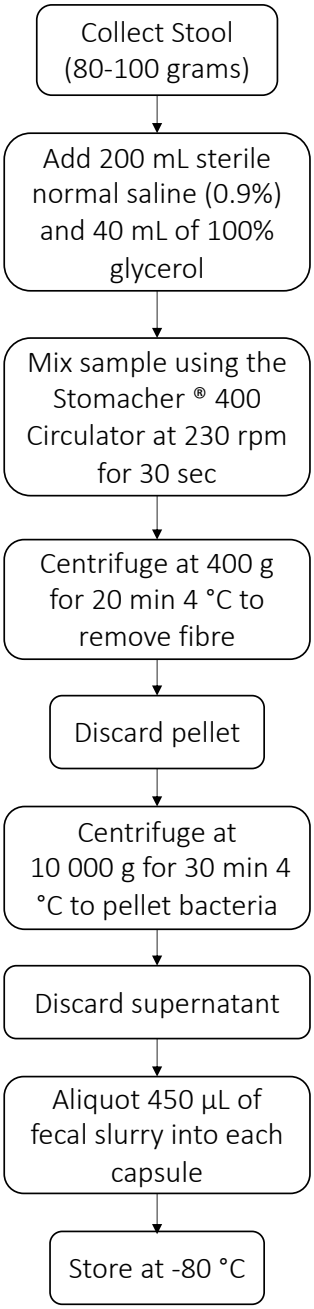

Figure 3.1 Methodology for the formation of FMT capsules.

3.2.3 Bacterial Culture

Samples were tested at initial sample collection, immediately after the encapsulation process, 1 day, 3 days, 5 days, 7 days, 2 weeks, 1 month, and 2 months' post-encapsulation. At each time point three capsules from each donor were thawed and dissolved in 10 mL of

pre-reduced sterile PBS inside of an anaerobic chamber. The sample was serially diluted in PBS and plated on 5 different agar plates: BHIS agar (BD, Mississauga, ON) (selective for total anaerobes), MacConkey agar (BD) (selective for *Enterobacteriaceae*), CBA (BD) (selective for anaerobic staphylococci and streptococci), Columbia CNA Blood agar (BD) (selective for aerobic staphylococci, streptococci and enterococci), and Rogosa agar (BD) (selective for lactobacilli and bifidobacteria). BHIS, Rogosa, and CBA plates were incubated at 37 °C for 48 hours in an anaerobic chamber. Columbia CNA and MacConkey agar plates were incubated at 37 °C for 24 hours in aerobic conditions.

3.2.4 PMA Exposure

At each time point 2.5 µL of 20 mM propidium monoazide (PMA; Biotium, Scarborough, ON) was added to 500 µL of diluted capsule material in 1.5 mL Eppendorf tubes for a final concentration of 100 µM PMA. The samples were vortexed and then covered in tin foil and placed in the dark for 10 minutes. The samples were then placed lying flat on ice and were exposed to light for 15 minutes (650 watts) that was 30 cm above the samples in a cold room. The cells were spun at 10 000 *x* g for 10 minutes, the supernatant discarded and resuspended in nuclease free water three times. After the final spinning step, the sample was stored in 100 µL of nuclease free water and stored at -80 °C until time for DNA extraction.

3.2.5 DNA Extraction

DNA from the 100 µL cell pellets was extracted using the DNeasy® Powersoil® HTP 96 Kit (Qiagen, Toronto, Ontario, Canada), as per the manufacturer's instructions with the following modification: a centrifuge speed of 3 700 g for 10 minutes was used. Extracted DNA was stored at -20 °C until amplification.

3.2.6 DNA Amplification

The BioMek® 3000 Laboratory Automation Workstation for automated PCR reagent set up was used to load 10 µL (2.3 pmol/µL) of 32 primers (16 left and 16 right) with unique

barcodes into 96 well plates. Amplifications of the V4 region of the 16S rRNA gene were carried out with the primers (5′-3′) ACACTCTTTCCCTACACGACGCTCTTCCGATCT

NNNNxxxxxxxxGTGCCAGCMGCCGCGGTAA and (5′-3′) CGGTCTCGGCATTCCT

GCTGAACCGCTCTTCCGATCTNNNNxxxxxxxxGGACTACHVGGGTWTCTAAT (xxxxxxxx is a sample specific nucleotide barcode and the preceding sequence is a portion of the Illumina adapter sequence for library construction). The BioMek® robot was then used to transfer 2 µL of template DNA into the primer containing 96 well plates. Then 20 µl of Promega GoTaq® Colourless Master Mix (Promega, Maddison, WI) was added to the DNA template and primers. The final plate was sealed with a foil PCR plate cover. This plate was placed in the Eppendorf Mastercycler® thermal cycler (Eppendorf, Mississauga, Ontario, Canada), where the lid was kept at 105 °C. An initial warm-up temperature of 95 °C was used for 2 min to activate the GoTaq®. Afterwards, the volumes underwent 25 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. After completion, the temperature of the thermal cycler was held at 4 °C, and amplicons were stored at −20 °C.

3.2.7 DNA sequencing and data analysis

Amplified DNA was sent to the London Regional Genomics Centre at Robarts Research Institute (Western University, London, Ontario, Canada). The samples were quantified (Quant-it, Life Technologies, Burlington, Ontario, Canada) and pooled at equimolar concentrations. The pooled libraries were cleaned using QIAquick (Qiagen, Germantown, Maryland, USA) and then sequenced using the MiSeq Illumina® platform, with 2 × 250 bp paired-end chemistry. The reads were demultiplexed and filtered using dada2 (version 1.8) and custom R scripts written by Greg Gloor (github.com/ggloor/miseq_bin). The demultiplexed reads will be available at NCBI SRA following submission of this paper for publication. Reads with 97% sequence similarity were grouped into OTUs. Taxonomy was assigned using an RDP classifier provided by the dada2 package and trained against version 132 of the SILVA database. Diversity of the stool microbiota was quantified based on Shannon Diversity Index and was calculated using the Vegan package

(github.com/vegandevs/vegan) in R (version 3.6.0). Bar plots and dendrogram were constructed in base R. OTUs with the same genus were grouped together in the bar plot. The 30 most prevalent genera are displayed in the bar plot. DNA extracted from capsules stored for 2 months from Donor 1 and stored for 2 weeks from Donor 2 were not adequate for sequencing and were not included in this analysis.

3.2.8 Statistical Analysis:

Bacterial viability data and Shannon diversity index passed the Shapiro-Wilk test for normality. Levene's test of homogeneity was significant and as a result One-way ANOVA was used to compare bacterial viability of fresh samples to subsequent time points as well as Shannon Diversity Index. Dunnett's multiple comparisons test, with a single pooled variance, was used post-hoc. One-way ANOVA was used to compare differences in the concentration of viable bacteria as well as Shannon Diversity Index between donors at each time point. Tukey's multiple comparison test, with a single pooled variance, was used post-hoc. The ALDEx2 package in R[24] was used to identify differentially abundant taxa with an effect size cutoff of > |3| to determine what bacterial taxa were differentially abundant from fresh stool that had not been treated with PMA at each time point. Effect size was used to identify differentially abundant OTUs, instead of a p-value, because effect size measures the magnitude of change and it is not affected by sample size[25].

3.3 Results

3.3.1 Significant reductions in bacterial viability occurred during encapsulation

FMT capsules were manufactured and changes in bacterial viability during preparation and storage at -80 °C were measured using culture-based techniques (Figure 3.2). The largest decrease in the viability of bacteria occurred during the encapsulation process (Figure 3.2). All three donors did not differ in the concentration of total anaerobes present in fresh stool, before encapsulation (Figure 3.2a). There were significant decreases in the concentration of viable total anaerobes, Gram-positive anaerobes, lactobacilli, and Gram-positive aerobes

in all three donors following encapsulation (Figure 3.2a, b, c, f). Stool from Donor 3 was the only sample to experience a significant decrease in bifidobacteria immediately following encapsulation (Figure 3.2d). Stool from Donor 2 was the only sample to experience a significant decrease in Gram-negative aerobes immediately following encapsulation (Figure 3.2e). The concentration of viable bacteria from all bacterial groups remained stable following encapsulation during storage at -80 °C (Figure 3.2). Statistics for only the comparison of the concentration of viable bacteria in fresh stool versus freshly made capsules were included in Figure 3.2. If there was a decrease in concentration after the capsules were made, this change persisted for subsequent time points. The full summary of statistics can be found in Supplementary Tables 3.1-3.2 (Figure 3.2a), Supplementary Tables 3.3-3.4 (Figure 3.2b), Supplementary Tables 3.5-3.6 (Figure 3.2c), Supplementary Tables 3.7-3.8 (Figure 3.2d), Supplementary Tables 3.9-3.10 (Figure 3.2e), Supplementary Tables 3.11-3.12 (Figure 3.2f).

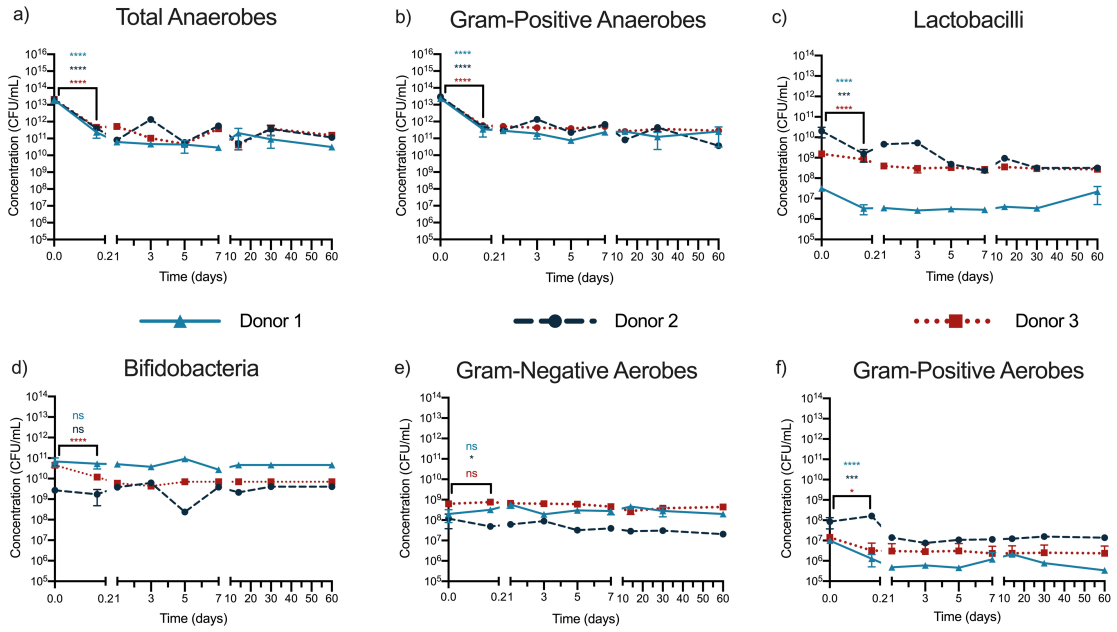

Figure 3.2 Bacterial viability was stable throughout storage in FMT capsules. Eighty to one hundred grams of stool was mixed with 200 mL of saline and 40 mL of 100% glycerol and spun at 10 000g for 30 minutes. The cell pellet was collected and 450 µL of

pellet was used to fill each FMT capsule. Capsules were stored at -80 °C. At each time point (immediately after collection, immediately after encapsulation, 1 day, 3 days, 5 days, 1 week, 2 weeks, 4 weeks, and 8 weeks) three 1 g aliquots or 3 capsules from each donor were thawed and plated on selective media in aerobic and anaerobic conditions and incubated at 37 °C for 24-48 hours. Data displayed is three biological replicates (n=3) and each biological replicate had six technical replicates. One-way ANOVA was used to compare differences in the concentration of different bacterial groups at baseline (fresh stool) to subsequent time points. This analysis was completed for each individual donor. A detailed summary of statistics including the results of the post-hoc tests can be found in Supplementary Tables 1-12. Results of the post-hoc test to determine if there was a significant change in the concentration of viable bacteria of fresh stool compared to freshly made capsules are listed below.

A) Total anaerobes. Donor 1: ****$p<0.0001$; Donor 2 ****$p<0.0001$; Donor 3 ****$p<0.0001$.
B) Gram-positive anaerobes. Donor 1: ****$p<0.0001$; Donor 2: ****$p<0.0001$; Donor: 3 ****$p<0.0001$.
C) Lactobacilli. Donor 1: ****$p<0.0001$; Donor 2: ***$p=0.0002$; Donor 3: ****$p<0.0001$.
D) Bifidobacteria. Donor 1: $p=0.8050$; Donor 2: $p=0.6683$; Donor 3: ****$p<0.0001$.
E) Gram-negative aerobes. Donor 1: $p=0.6474$; Donor 2: *$p=0.0362$; Donor 3: $p=0.3978$.
F) Gram-positive aerobes. Donor 1: ****$p<0.0001$; Donor 2: ***$p=0.0005$; Donor 3: *$p=0.0121$.

3.3.2 Fecal microbiota profiles of donors are distinct throughout storage.

Since there were changes in the concentration of viable bacteria in the FMT capsules, we wanted to determine if the donor microbiota composition was still distinct from other donors using PMA and next-generation sequencing. A dendrogram was constructed to

group fecal samples based on compositional similarity. Individual donors clustered separately regardless of the duration of storage or if the encapsulation process had taken place (Figure 3.3).

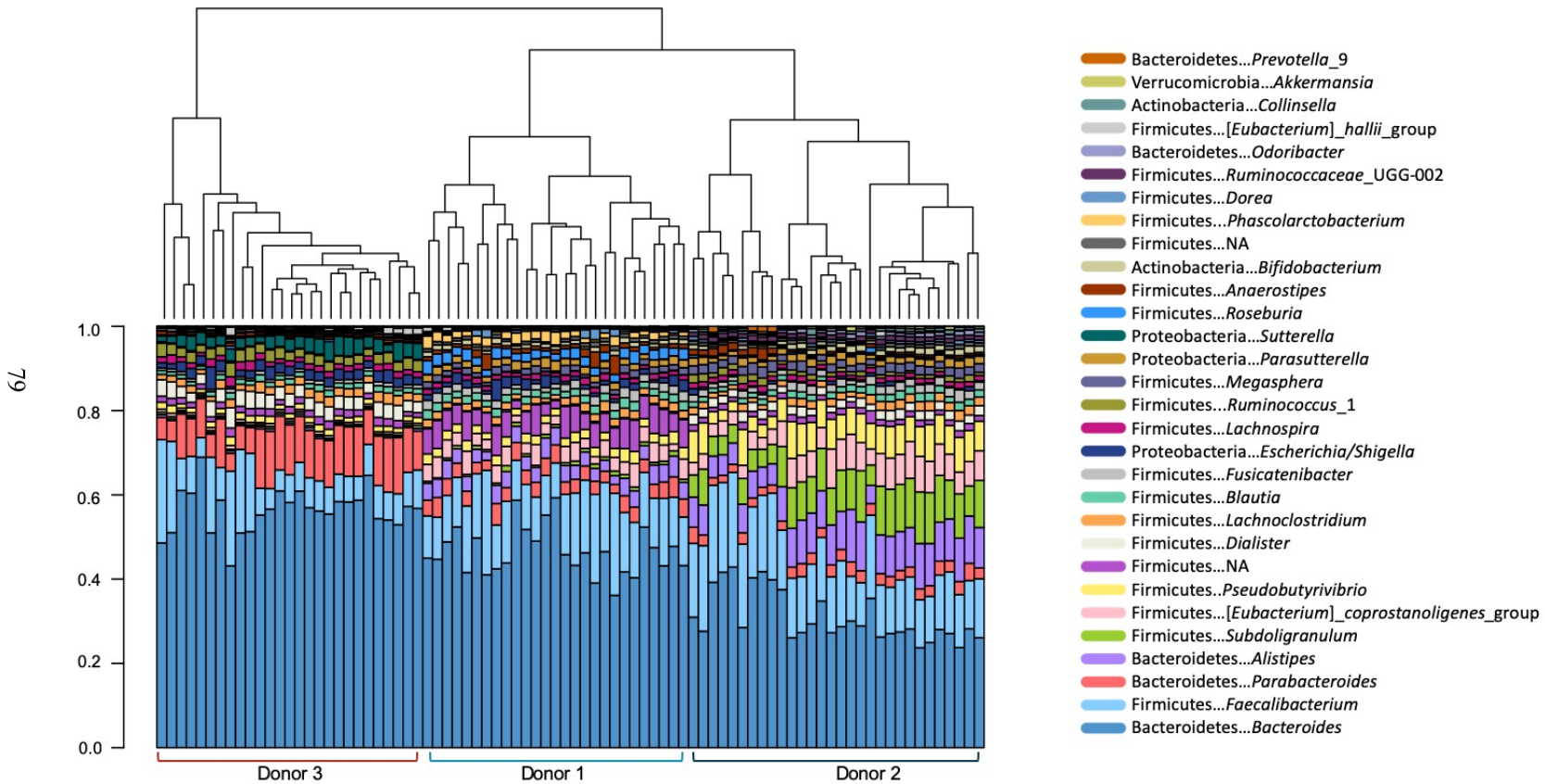
1.0
0.8
0.6
0.4
0.2
0.0
Donor 3
Donor 1
Donor 2
Bacteroidetes...Prevotella_9
Verrucomicrobia...Akkermansia
Actinobacteria...Collinsella
Firmicutes...[Eubacterium]_hallii_group
Bacteroidetes...Odoribacter
Firmicutes...Ruminococcaceae_UGG-002
Firmicutes...Dorea
Firmicutes...Phascolarctobacterium
Firmicutes...NA
Actinobacteria...Bifidobacterium
Firmicutes...Anaerostipes
Firmicutes...Roseburia
Proteobacteria...Sutterella
Proteobacteria...Parasutterella
Firmicutes...Megasphera
Firmicutes...Ruminococcus_1
Firmicutes...Lachnospira
Proteobacteria...Escherichia/Shigella
Firmicutes...Fusicatenibacter
Firmicutes...Blautia
Firmicutes...Lachnoclostridium
Firmicutes...Dialister
Firmicutes...NA
Firmicutes..Pseudobutyrivibrio
Firmicutes...[Eubacterium]_coprostanoligenes_group
Firmicutes...Subdoligranulum
Bacteroidetes...Alistipes
Bacteroidetes...Parabacteroides
Firmicutes...Faecalibacterium
Bacteroidetes...Bacteroides

Figure 3.3 The composition of viable bacteria was stable throughout storage and each donor microbiota profile remained distinct from one another. Eighty to one hundred grams of stool was mixed with 200 mL of saline and 40 mL of 100% glycerol and spun at 10 000g for 30 minutes. The cell pellet was collected and 450 µL of pellet was used to fill each FMT capsule. Capsules were stored at -80 °C. At each time point (immediately after collection, immediately after encapsulation, 1 day, 3 days, 5 days, 1 week, 2 weeks, 1 month, and 2 months) three 1 g aliquots or 3 capsules from each donor were thawed and treated with PMA under anaerobic conditions and sent for next-generations sequencing. The resulting reads were used to generate a bar plot and dendrogram in R (version 3.6.0). The bar plot was ordered by the dendrogram grouping. Donor 1 is missing data points for 2 months storage and Donor 3 is missing data points for 2 weeks storage because the samples were not adequate for sequencing. The three donors had distinct viable microbiota compositions throughout storage.

This was confirmed with a principal component analysis (PCA) that showed that the three individual donors have unique microbiota compositions and the microbiota profiles of a donor remained distinct from other donors irrespective of the duration of storage (Figure 3.4). The distance between two points on a PCA is representative of their similarity in composition. Principal components 1 and 2 account for 67.3% of the variance explained.

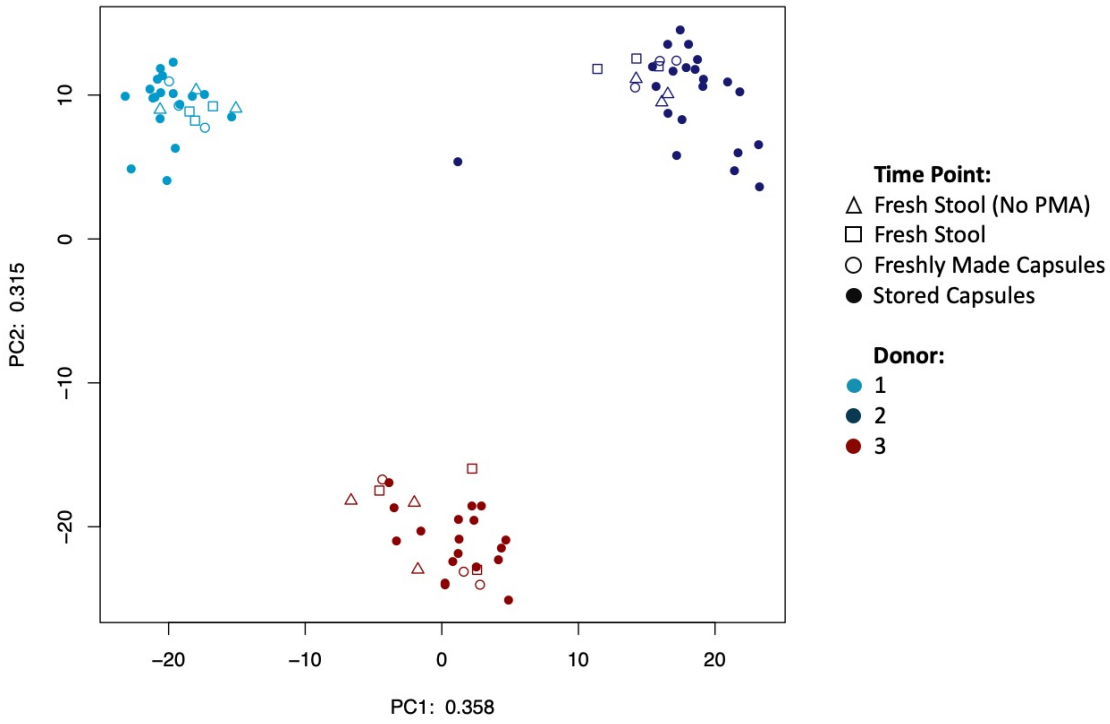

Figure 3.4 Each donor microbiota profile remained distinct from one another throughout storage. Eighty to one hundred grams of stool was mixed with 200 mL of saline and 40 mL of 100% glycerol and spun at 10 000g for 30 minutes. The cell pellet was collected and 450 µL of pellet was used to fill each FMT capsule. Capsules were stored at -80 °C. At each time point (immediately after collection, immediately after encapsulation, 1 day, 3 days, 5 days, 1 week, 2 weeks, 1 month, and 2 months) three 1 g aliquots or 3 capsules from each donor were thawed and treated with PMA under anaerobic conditions and sent for next-generations sequencing. The resulting reads were used to generate a PCoA in R (version 3.6.0) with custom R scripts written by Greg Gloor (github.com/ggloor/miseq_bin) and the package zCompositions. Sixty-nine percent of the variance is explained in the first two components. Each point represents the composition of one FMT capsule (each time point has three technical replicates that are displayed). The donors cluster separately from one another regardless of the duration of storage. Donor 1 is missing data points for 2 months storage and Donor 3 is missing data points for 2 weeks storage because the samples were not adequate for sequencing.

3.3.3 Fecal diversity was stable throughout storage

Since there were significant reductions of the viable bacteria in the FMT capsules, diversity was measured to determine if the loss of viability related to a change in diversity. Diversity was measured using Shannon Diversity Index and differences in diversity between fresh stool and stored capsules were calculated. Initial diversity varied between donors (Figure 3.5). Donor 1 had an initial fecal diversity of 3.61 ± 0.11, Donor 2: 3.55 ± 0.09 and Donor 3: 3.12 ± 0.20. Changes in diversity over time were different for each donor. Stool from Donor 3 did not exhibit any significant change in diversity of viable bacteria during encapsulation or storage (Figure 3.5). Stool from Donor 1 had a significant decrease in diversity immediately after encapsulation, but this effect was not observed during storage at -80 °C (Figure 3.5). Stool from Donor 2 experienced significant increases in diversity immediately after encapsulation, 1 day, 1 week, 2 weeks, 1 month and 2 months of storage (Figure 3.5).

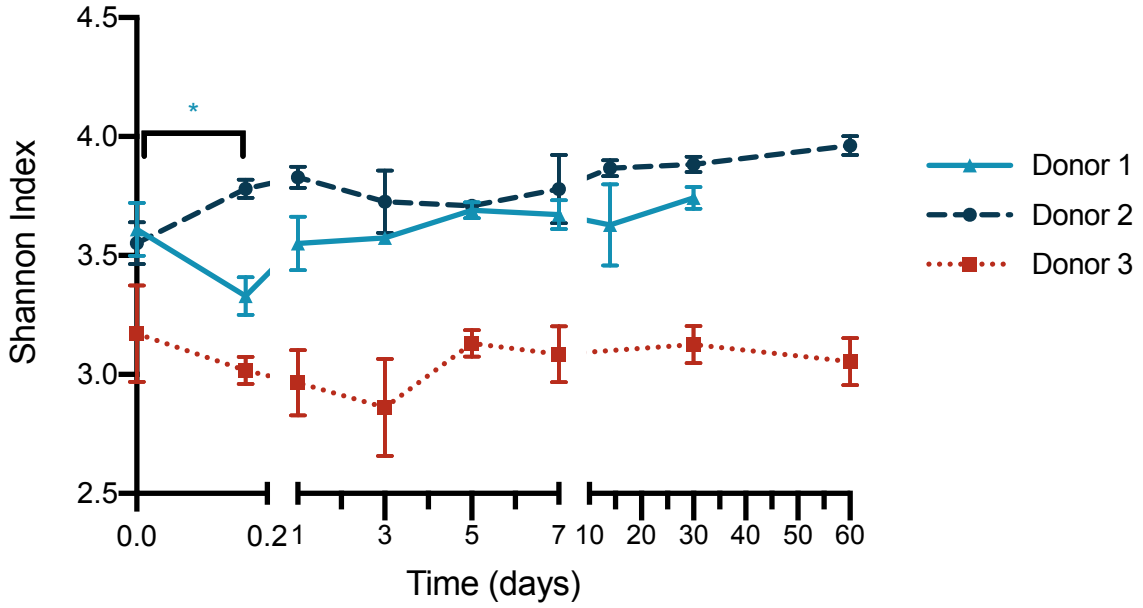

Figure 3.5 Diversity of donor stool was maintained through storage in FMT capsules. Eighty to one hundred grams of stool was mixed with 200 mL of saline and 40 mL of 100% glycerol and spun at 10 000 *x* g for 30 minutes. The cell pellet was collected and 450 µL

of pellet was used to fill each FMT capsule. Capsules were stored at -80 °C. At each time point (immediately after collection, immediately after encapsulation, 1 day, 3 days, 5 days, 1 week, 2 weeks, 1 month, and 2 months) three 1 g aliquots or 3 capsules from each donor were thawed and treated with PMA under anaerobic conditions and sent for next-generations sequencing. Shannon diversity index was calculated on the resulting sequencing reads. Each data point is the average of three technical replicates. Donor 1 is missing data points for 2 months storage and Donor 3 is missing data points for 2 weeks storage because the samples were not adequate for sequencing. One-way ANOVA was used to compare changes in Shannon Diversity Index of fresh stool samples to subsequent time points. A summary of statistics including the result of post-hoc tests are included in Supplementary Tables 3.13-3.14. This analysis was completed for each individual donor. Only significant decreases in diversity are shown on the graph. Donor 1: *p=0.0103. No other comparisons of fresh stool to subsequent time points resulted in significant decreases in diversity for any of the three donors.

3.3.4 Viable fecal microbiota composition varied by donor throughout encapsulation and storage.

The largest decrease in viable bacteria occurred during the encapsulation process and remained stable throughout storage. There was not a reduction in diversity of viable bacteria inside of the capsules following encapsulation, but it was not yet known what specific genera may have changes in relative abundance. The ALDEx2 tool was used to determine the differentially abundant bacteria that increased or decreased in relative abundance throughout encapsulation and storage for each donor. Each donor had a different change in viable bacterial composition throughout processing and storage (Table 3.1).

During initial sample collection, the fecal sample from Donor 1 had a decrease in the relative abundance of viable *Anaerostipes* and increases in the relative abundances of viable *Bacteroides, Parasutterella,* and *Roseburia* (Table 3.1; Figure 3.6). Donors 2 and 3 had no significant changes in the relative abundances of viable bacteria during sample collection (Table 3.1; Figure 3.6). The composition of viable bacteria was examined

immediately after the stool was formed into capsules. Donor 1 showed decreases in the relative abundances of viable *Anaerostipes* and *Faecalibacterium* and increases in the relative abundances of viable *Alistipes, Bacteroides, Escherichia/Shigella* and *Parasutterella* (Table 3.1; Figure 3.6). Encapsulated stool from Donor 2 showed decreases in the relative abundances of viable *Anaerostipes, Bacteroides, Faecalibacterium* and *Ruminococcus*_1 and increases in the relative abundances of viable *Alistipes* and *Pseudobutyrivibrio* (Table 3.1; Figure 3.6). Donor 3 exhibited no changes in the composition of viable bacteria following the encapsulation process (Table 3.1; Figure 3.6).

Following this, the capsules were frozen at -80 °C and three capsules from each donor were thawed after 24 hours to examine the changes in viable bacteria that may occur during the freeze-thaw of the capsules when used in clinics. Capsules from Donor 1 had decreases in the relative abundances of viable *Alistipes*, *Blautia,* and *Escherichia/Shigella* and increases in the relative abundances of viable *Anaerostipes* and *Dorea* (Table 3.1; Figure 3.6). Capsules from Donor 2 had a decrease in the relative abundances of viable *Bifidobacterium, Pseudobutryrivibrio* and increases in the relative abudnances of viable *Anaerostipes, Bacteroides, Faecalibacterium, Lachnospira, Ruminococcus*_1*,* and *Lachnoclostridium* (Table 3.1; Figure 3.6). Capsules from Donor 3 showed no significant changes in the composition of viable bacteria following the freeze-thaw (Table 3.1; Figure 3.6).

Table 3.1 Summary of differentially abundant genera of encapsulated stool compared to unprocessed stool.

	Donor 1		Donor 2		Donor 3	
	Change in Relative Abundance	**Effect Size**	**Change in Relative Abundance**	**Effect Size**	**Change in Relative Abundance**	**Effect Size**
Fresh	↓ *Anaerostipes*	-3.008	No Change		No Change	
	↑ *Bacteroides*	3.582				
	↑ *Bacteroides*	3.072				
	↑ *Bacteroides*	3.893				
	↑ *Parasutterella*	3.806				
	↑ *Roseburia*	3.243				
Capsules	↓ *Anaerostipes*	-4.647	↓ *Anaerostipes*	-3.371	No Change	
	↓ *Faecalibacterium*	-4.629	↓ *Bacteroides*	-3.313		
	↓ *Faecalibacterium*	-3.420	↓ *Bacteroides*	-4.321		
	↓ *Faecalibacterium*	-3.251	↓ *Bacteroides*	-5.576		
	↑ *Alistipes*	3.322	↓ *Bacteroides*	-3.311		
	↑ *Bacteroides*	3.150	↓ *Bacteroides*	-5.067		
	↑ *Bacteroides*	3.069	↓ *Bacteroides*	-9.330		
	↑ *Bacteroides*	3.767	↓ *Bacteroides*	-7.467		
	↑ *Bacteroides*	3.551	↓ *Faecalibacterium*	-3.240		
	↑ *Bacteroides*	4.876	↓ *Ruminoccocus_1*	-3.095		
	↑ *Bacteroides*	3.075	↑ *Alistipes*	3.814		
	↑ *Bacteroides*	6.909	↑ *Pseudobutyrivibrio*	3.727		
	↑ *Escherichia/Shigella*	7.775				
	↑ *Parasutterella*	5.612				
1 day	↓ *Alistipes*	-3.229	↓ *Bifidobacterium*	-3.359	No Change	
	↓ *Blautia*	-3.400	↓ *Pseudobutyrivibrio*	-3.621		
	↓ *Escherichia/Shigella*	-4.231	↑ *Anaerostipes*	6.8423		
	↑ *Anaerostipes*	6.393	↑ *Bacteroides*	4.200		
	↑ *Dorea*	3.927	↑ *Bacteroides*	4.535		
			↑ *Bacteroides*	3.343		
			↑ *Bacteroides*	5.359		
			↑ *Bacteroides*	3.289		
			↑ *Bacteroides*	7.753		
			↑ *Bacteroides*	6.746		
			↑ *Bacteroides*	4.991		
			↑ *Faecalibacterium*	5.170		
			↑ *Faecalibacterium*	4.181		
			↑ *Faecalibacterium*	3.577		
			↑ *Faecalibacterium*	3.285		
			↑ *Lachnospira*	3.432		
			↑ *Ruminococcus_1*	4.133		
			↑ *Lachnoclostridium*	4.441		
3 days	↑ *Anaerostipes*	6.312	↓ *Bacteroides*	-5.725	No Change	
	↑ *Dorea*	6.692	↑ *Anaerostipes*	6.625		
			↑ *Bacteroides*	3.688		
			↑ *Bacteroides*	3.417		
			↑ *Eubacterium*	3.292		
			↑ *Faecalibacterium*	3.876		
			↑ *Faecalibacterium*	3.857		

			↑ *Ruminococcus_1*	3.372		
5 days	↓ *Escherichia/Shigella* ↑ *Anaerostipes* ↑ *Dorea* ↑ *Lachnoclostridium*	-5.110 5.641 4.246 3.012	↓ *Pseudobutyrivibrio* ↑ *Bacteroides* ↑ *Ruminococcus_1*	-3.597 4.296 3.290	No Change	
1 week	↓ *Anaerostipes* ↓ *Dorea*	-7.621 -4.066	↓ *Anaerostipes* ↓ *Bacteroides* ↓ *Faecalibacterium* ↓ *Faecalibacterium* ↓ *Lachnoclostridium* ↑ *Bifidobacterium* ↑ *Pseudobutyrivibrio* ↑ *Subdoligranulum*	-9.437 -4.558 -4.974 -3.003 -3.805 4.050 3.094 3.083	No Change	
2 weeks	↓ *Anaerostipes* ↓ *Dorea* ↑ *Blautia* ↑ *Fusicantenibacter*	-9.478 -5.714 4.385 3.039	↓ *Anaerostipes* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Lachnoclostridium* ↑ *Bifidobacterium* ↑ *Eubacterium* ↑ *Pseudobutyrivibrio* ↑ *Subdoligranulum*	-6.981 -3.357 -4.361 -3.674 3.251 3.970 3.573 3.235	NA	NA
1 month	↓ *Anaerostipes* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bifidobacterium* ↓ *Faecalibacterium* ↓ *Faecalibacterium* ↓ *Faecalibacterium* ↓ *Faecalibacterium* ↓ *Faecalibacterium* ↓ *Lachnoclostridium* ↓ *Parasutterella* ↓ *Romboutsia* ↓ *Subdoligranulum* ↑ *Prevotella_9*	-14.833 -3.195 -3.587 -3.139 -11.509 -5.475 -8.867 -6.147 -5.789 -3.611 -3.482 -3.239 -3.102 3.320	↓ *Anaerostipes* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Dialster* ↓ *Faecalibacterium* ↓ *Faecalibacterium* ↓ *Faecalibacterium* ↓ *Lachnoclostridium* ↓ *Lachnospira* ↓ *Ruminococcus_1* ↓ *Parabacteroides* ↓ *Parabacteroides* ↓ *Sutterella* ↑ *Bifidobacterium* ↑ *Subdoligranulum*	-8.868 -6.291 -3.241 -3.769 -3.001 -16.163 -4.957 -3.677 -4.669 -8.467 -5.188 -6.418 -4.317 -3.762 -5.424 -3.162 -3.309 -5.211 3.466 3.065	No Change	
2 months	NA	NA	↓ *Anaerostipes* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides* ↓ *Bacteroides*	-5.556 -4.292 -4.311 -3.239 -4.056 -7.115	No Change	

			↓ *Bacteroides*	-7.639		
			↓ *Bacteroides*	-4.326		
			↓ *Bacteroides*	-4.164		
			↓ *Eubacterium*	-3.496		
			↓ *Faecalibacterium*	-5.443		
			↓ *Faecalibacterium*	-5.820		
			↓ *Faecalibacterium*	-4.975		
			↓ *Faecalibacterium*	-6.585		
			↓ *Lachnoclostridium*	-3.277		
			↓ *Lachnospira*	-3.171		
			↓ *Roseburia*	-3.224		
			↓ *Ruminococcus*_1	-4.175		

Differentially abundant genera were determined using ALDEx2 (Fernandes et al. 2013) and fresh samples without PMA treatment were compared to subsequent time points. An effect size > |3| was considered a significant change in relative abundance. There were multiple OTUs that had the same genus but were similar enough in sequence to be sorted into the same OTUs.

NA: sequencing data not available

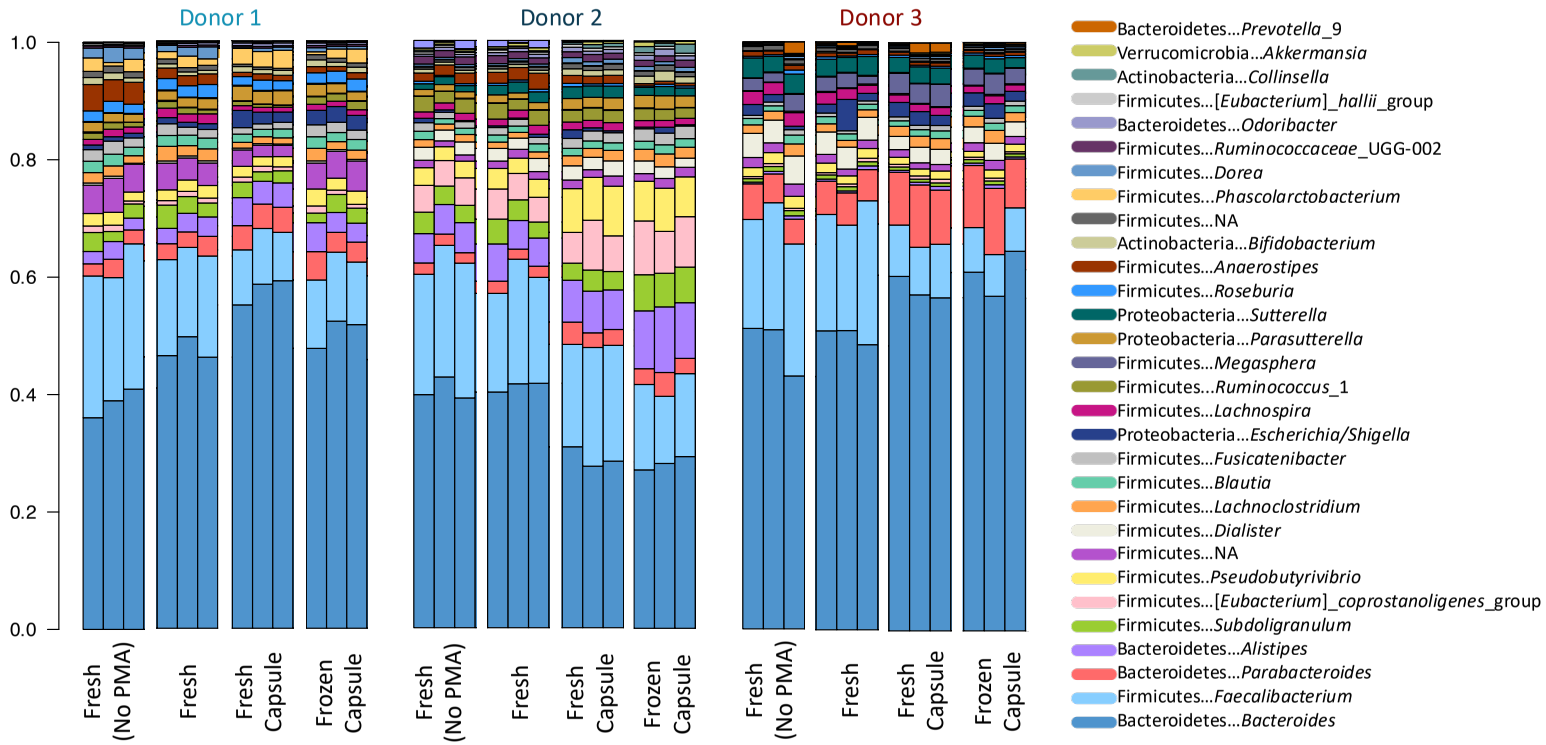
Donor 1
Donor 2
Donor 3
1.0
0.8
0.6
0.4
0.2
0.0
Fresh (No PMA)
Fresh
Fresh Capsule
Frozen Capsule
Bacteroidetes...Prevotella_9
Verrucomicrobia...Akkermansia
Actinobacteria...Collinsella
Firmicutes...[Eubacterium]_hallii_group
Bacteroidetes...Odoribacter
Firmicutes...Ruminococcaceae_UGG-002
Firmicutes...Dorea
Firmicutes...Phascolarctobacterium
Firmicutes...NA
Actinobacteria...Bifidobacterium
Firmicutes...Anaerostipes
Firmicutes...Roseburia
Proteobacteria...Sutterella
Proteobacteria...Parasutterella
Firmicutes...Megasphera
Firmicutes...Ruminococcus_1
Firmicutes...Lachnospira
Proteobacteria...Escherichia/Shigella
Firmicutes...Fusicatenibacter
Firmicutes...Blautia
Firmicutes...Lachnoclostridium
Firmicutes...Dialister
Firmicutes...NA
Firmicutes..Pseudobutyrivibrio
Firmicutes...[Eubacterium]_coprostanoligenes_group
Firmicutes...Subdoligranulum
Bacteroidetes...Alistipes
Bacteroidetes...Parabacteroides
Firmicutes...Faecalibacterium
Bacteroidetes...Bacteroides

Figure 3.6 Changes in the composition of viable bacteria during the sample collection and encapsulation process. Eighty to one hundred grams of stool was mixed with 200 mL of saline and 40 mL of 100% glycerol and spun at 10 000 *x* g for 30 minutes. The cell pellet was collected and 450 µL of pellet was used to fill each FMT capsule. Samples displayed in this figures are three aliquots of the fresh stool collected from donors without PMA treatment (Fresh [No PMA]), three aliquots of fresh stool collected from donors with PMA treatment (Fresh), three freshly prepared capsules from each donor (Fresh Capsule), and three capsules that had been frozen for 24 hours (Frozen Capsule). The DNA was extracted from these samples and sequenced using the Illumina Mi-Seq. The resulting reads were used to generate bar plots in R (version 3.6.0).

The capsules were stored for 2 months to simulate the duration of storage they would likely experience in an FMT clinic. Donor 3 did not have any significant changes to the composition of viable bacteria throughout the storage process. Capsules made from stool from Donors 1 and 2 did undergo changes in the composition of viable bacteria during long-term storage. Capsules from both donors had decreases in the relative abundances of viable *Anaerostipes, Bacteroides, Faecalibacterium,* and *Lachnoclostridium* following one and two months of storage (Table 3.1).

3.4 Discussion

FMT capsules are quickly replacing conventional FMT delivery methods, such as enema, colonoscopy, or nasojejunal tube. It is not known how this different manufacturing technique impacts the concentration and composition of viable microbes delivered during FMT. This was the first study to investigate the composition of viable bacteria in FMT capsules with bacterial culture and PMA next-generation sequencing. We have shown that FMT capsule preparation caused a reduction in viable bacteria during the encapsulation phase and that FMT capsules were stable during long-term storage.

The largest decrease in viable bacteria occurred during the manufacturing of the FMT capsules (Figure 3.2). Capsules from Donor 1 lost viable *Anaerostipes and*

Faecalibacterium and capsules from Donor 2 lost viable *Anaerostipes, Bacteroides, Faecalibacterium,* and Ruminococcus_1 (Table 3.1; Figure 3.6). FMT capsules take longer to prepare compared to enemas. In our clinic, it takes approximately an hour and fifteen minutes to thaw and prepare a fecal sample for an enema delivered FMT, whereas the process of manufacturing FMT capsules takes approximately four hours and involves much more processing and handling. The increase in processing time and duration of oxygen exposure may be responsible for the decline in viability that we observed during the encapsulation process, as two studies have shown that oxygen exposure has the greatest effect on the composition of viable bacteria in donor stool [20,22]. Another potential cause for the reduction in viable bacteria could be that the dietary fibre is removed during the encapsulation process and any bacteria bound to this would have also been removed. It is necessary to remove fibre during the encapsulation process to prevent obstructions in the pipettes used to fill the capsules and to ensure that each capsule contains the same concentration of bacteria.

Each capsule on average contained approximately 1.0×10^{11} viable bacteria after two months of storage at -80 °C (Figure 3.2). At our clinic, a single dose consists of 40 capsules, therefore patients receive approximately 4.0×10^{12} bacteria in one dose. This is comparable to other studies that have published the number of viable bacteria in a single FMT delivered by capsule at their clinics. Hirsch et al.[10] estimated that one FMT dose was 9.7×10^{10} viable bacteria in 10 capsules. Staley et al.[26] estimated $2.1\text{-}2.5 \times 10^{11}$ bacteria were delivered in 2-3 capsules. Kao et al.[9] estimated that approximately 10^{13} bacteria were delivered in one dose of 40 capsules. While there is a range of viable bacteria delivered in different studies, the clinical resolution rates of rCDI remain similar. Hirsch et al.[10] achieved a resolution rate of 89% (17/19), Staley et al.[26] achieved a resolution rate of 93.3% (28/30), and Kao et al.[9] achieved a resolution rate of 96.2 % (51/53. Kao et al.[9] also treated a group of rCDI patients with colonoscopy delivered FMT and found that 96.2 % (50/52) of patients achieved clinical resolution, proving that capsules were just as effective at treating rCDI as traditional FMT delivery methods.

There were some significant changes in the composition of viable bacteria during storage and we found that each donor experienced different changes over time (Table 3.1). Donors 1 and 2 had a decrease in the relative abundance of *Faecalibacterium* and two studies have also found that *Faecalibacterium* is lost during oxygen exposure[20,22] because it is a strict anaerobe. *Faecalibacterium prausnitzii,* a known butyrate producer, has been shown to promote the release of anti-inflammatory cytokines[27], and could potentially be protective against obesity and insulin resistance[28]. The loss of this genus may cause capsule based FMTs to be less effective for treating obesity-related disorders. However, there have been no clinical trials to support or oppose this. *Anaerostipes* decreased in relative abundance in both Donors 1 and 2. Species of *Anaerostipes* are also known butyrate producers[29,30] and a decrease in *Anaerostipes* has been found to be associated with irritable bowel syndrome and colorectal cancer[31,32].

The differences that occurred over time within each donor were significantly smaller than the differences in composition between donors. When samples were clustered in a dendrogram and a PCA based on their similarity in composition, we saw that each individual donor clustered separately, regardless of the duration of storage (Figures 3.3, 3.4). Maintaining the composition of viable microbes is important as an altered microbial composition could pass along the phenotype of undesired conditions or lack the bacteria necessary for a successful FMT. Only one of the three donors experienced a decrease in fecal bacterial diversity during encapsulation and storage. (Figure 3.5). This finding is particularly important because increased fecal microbiota diversity has been shown to be associated with resolution of and protection against rCDI[9,33,34], and FMT material with lower bacterial diversity may have decreased efficacy. Stool from Donor 2 had an increase in diversity during storage (Figure 3.5), which likely occurred as an artefact of PMA treatment. Donor 2 had a decrease in the relative abundance viable *Bacteroides* over time, and this created more bacterial evenness as other bacterial genera appeared to increase in relative abundance, which likely contributed to the apparent rise in diversity.

FMT via capsule delivery has a number of advantages. Delivering FMT by capsule is cheaper than colonoscopy with the average capsule treatment costing $395 CAD versus $1120 for colonoscopy[9]. Capsules are a less invasive delivery method than enema or colonoscopy. Kao et al.[9] found that 66% of participants that received an FMT in the form of capsules rated the experience as not at all unpleasant versus 44% of participants who received an FMT in the form of colonoscopy.

The use of FMT capsules is relatively recent and we believe that there are a variety of options to improve the manufacturing of FMT capsules; however, they may be met with additional difficulties. Preparation of capsules in anaerobic conditions may prevent some of the reduction in viable bacteria that occurs during the encapsulation process. This might be difficult to achieve as anaerobic chambers are often limited in their size and this process requires a centrifuge. There is also a decrease in dexterity when working in an anaerobic chamber and this would likely increase the processing time of capsules. Alternatively, whole stool could be lyophilized and ground into a powder to fill FMT capsules[26,35]. Fibre would not have to be removed from the stool for lyophilization for which bacteria are known to strongly be associated with and adhere to this material. Additionally, the freeze-dried material would likely have a longer shelf life than the liquid filled FMT capsules and could potentially be stored without refrigeration, making it more accessible globally. The cost of purchasing and maintaining an anaerobic chamber or lyophilizer may be a limitation for a number of clinics that manufacture their own FMT material.

This study was the first to examine changes in the composition of viable bacteria in FMT capsules during encapsulation and storage. Strengths of this study include that we studied multiple donors as past studies have shown that survival of microbes in stool differs by donor[21,22]. Three capsules at each time point were examined to account for any variation in composition or concentration of viable bacteria between capsules. One of the limitations of this study was that not all bacteria are culturable. While a variety of selective media, and anaerobic and aerobic conditions were used to enumerate viable bacteria, the total number of viable bacteria that were present may have been underestimated. We have tried to

overcome this by using 16S rRNA gene analysis combined with PMA to sequester DNA from dead bacteria and free DNA. While the focus of this study was on the viable bacteria present in the capsules, past studies have shown that engraftment of the donor microbiota is not necessary for successful FMT[36], and that sterile FMTs[37], bacteriophage[38], and spores[39] have been shown to be associated with resolution of rCDI. There may be other components of FMT capsules beyond the viable bacteria that are delivered that are responsible for the resolution of rCDI.

The encapsulation of stool into FMT capsules and subsequent storage resulted in a significant loss of viable bacteria and while there were changes in the composition of viable bacteria in two of the three donors, the bacterial composition still closely resembled the initial composition of stool from each donor. Alternative methods for collecting and processing samples under anaerobic conditions should be investigated to prevent the initial bacterial loss that was experienced.

3.5 References

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Chapter 4

4 Extended screening costs associated with selecting donors for fecal microbiota transplantation for treatment of metabolic syndrome-associated diseases

4.1 Introduction

Fecal microbiota transplantation has been most commonly used to treat recurrent rCDI[1]. The impact of the gut microbiome on many other conditions and therefore the list of other potential indications for FMT, has been rapidly increasing[2]. Many aspects of human health, such as metabolism, autoimmune disease[3] and even mental health[4], are hypothesized to be impacted by the microbiome and FMTs are undergoing trials to assess their efficacy in many of these conditions[2].

The concerns regarding potential transmission of pathogenic organisms and thus the need for extensive pre-transplant donor-screening have been well known. To our knowledge there have been no reports of an infectious disease being transmitted through a screened FMT donor although there has been a report of possible cytomegalovirus transmission in an FMT from a non-screened donor[5]. However, the actual number of potential transmissible agents screened for has been a subject of practice variation and recently some authorities have released guidance documents to recommend minimum screening criteria[6]. A small number of clinical exclusion criteria for donation, such as recent antibiotic use, have been used for many years to improve the chances of success in prevention of recurrent *C. difficile*[7]. However, data on the myriad of potential diseases associated with the gut microbiome has steadily increased the number of conditions for which stool donation may carry a risk of transmission. The need to exclude donors with evidence of these conditions or even evidence of being at increased risk of having a microbiome associated with these conditions (such as having a family history of the disease, as the microbiome can be similar amongst family members that live together[8]) has left uncertainty regarding the cost and feasibility of donor screening. Therefore, we reviewed our experience in recently

establishing a new donor-screening program for FMTs for metabolic syndrome-related diseases and compared it to other groups screening processes and outcomes.

4.2 Methods

4.2.1 Donor Recruitment

Potential donors (PDs) were recruited through a hospital workplace, a university monthly newsletter, as well as by word of mouth amongst staff at St. Joseph's Health Care (a teaching hospital with over 4,000 employees, 2,000 residents and fellows, 1,100 physicians, and 1,000 health care student placements), Western University (with over 28,000 students, 2,400 employees, and 1,400 faculty members) and Lawson Health Research Institute (a hospital based research facility with over 1500 principal investigators, researchers, technicians, support staff and trainees distributed amongst ten sites) in London, Ontario, Canada. Recruitment took place from March 2015 to July 2016. The recruitment materials used listed a small number of the donor screening exclusion criteria including: BMI >25, abnormal metabolic profile, recent antibiotic use, family history of diabetes or coronary disease, and any known transmissible agent. PDs that expressed interest in being screened contacted the research coordinator to obtain more information on the screening process, to schedule screening, and provide written informed consent. A list of the full screening procedure and exclusion criteria was provided to PDs before screening was scheduled and those who believed that they would qualify proceeded to have a full history and examination by a physician.

4.2.2 Donor Screening

PDs written consent was obtained and medical histories and examinations of PDs were conducted by a physician. A summary of questions asked during the history and exam is detailed in Table 4.1. If the PD passed the initial screening criteria they were subjected to stool, urine and blood testing for transmissible diseases and other health markers. Donor screening practices of our program are outlined in Table 4.1. Lab screening was performed as per the Health Canada guidance document "Fecal microbiota therapy used in the

treatment of *Clostridium difficile* infection not responsive to standard therapies"[6].

In addition to testing for transmissible agents, laboratory screening included; screening for metabolic abnormalities (including HbA1C and fasting lipids), celiac disease (using anti-tissue transglutaminase antibodies), liver function tests and urinalysis. PDs were required to have: a healthy weight (BMI 18-25), no underlying conditions, a normal metabolic profile (no hypertension, normal fasting lipid profile), no history of injection drug use, no new sexual partners (within the last 3 months), no ongoing or recent use of any prescription or over the counter mediations (including antidiarrheal drugs, mineral oil, bismuth, magnesium or kaolin), a maximum alcohol intake of <10 g/day in women and <20 g/day in men, no recent antibiotic use (within 3 months) and no recent hospitalizations (within 3 months). No personal or family history of: diabetes, coronary disease or metabolic disease (hypertension, hyperlipidemia, diabetes, insulin insensitivity, atherosclerosis), gastrointestinal, liver or biliary disease (including: gastroesophageal reflux, peptic ulcer disease, celiac disease, inflammatory bowel disease, Crohn's disease, ulcerative colitis, microscopic colitis, or motility disorders). Those with previous surgery to the intestine, liver or gallbladder (except remote appendectomy) were also excluded. Any history of malignancy removed the PD for donation consideration.

PDs were screened for the following transmissible agents in blood: HIV type 1 and 2, Human T-cell lymphotropic virus 1 and 2, Hepatitis A, Hepatitis B, Hepatitis C, *Helicobacter pylori*, syphilis, *Stronglyoides,* schistosomiasis, amebiasis, cytomegalovirus (IgM), adenovirus, and Epstein Barr virus (IgM). Stool was analyzed for the detection of *Shigella, Salmonella, Yersinia, Campylobacter, Escherichia coli* 0157-H7, *Plesiomonas, Aeomonas,* Listeria*,* shiga toxins, ova, parasites, microsporidia, *C. difficile*, rotavirus, and norovirus. Pharyngeal and rectal swabs were assessed for gonococcal and chlamydia culture, as well as urine was assessed for the presence of gonorrhea and chlamydia by nucleic acid amplification tests. Nasal and rectal swabs were obtained to detect the presence of methicillin-resistant *Staphylococcus aureus* and the rectal swab was also assessed for the presence of vancomycin-resistant enterococci, extended spectrum beta-

lactamase producing *Enterobacteriaceae*, and carbapenem-resistant *Enterobacteriaceae*. If PDs had a history of travel to endemic areas, additional testing was performed for: Chagas disease, malaria, and babesiosis. If PDs traveled to Zika-endemic regions in the last 3 months, they were excluded. There were no PDs that had traveled to endemic areas and the additional testing was not performed. All laboratory test results were returned within 3 weeks' time and potential donors were asked to not engage in high risk behaviour after their screening took place.

Our donor screening methods were compared to 4 other programs, which have published their full screening methods and acceptance rates to illustrate the inconsistencies in screening PDs (Table 4.1).

The acceptance rates of these programs were contrasted with our own to determine what effect expanded screening-programs had on overall donor enrollment (Table 4.2). The cost of the screening program was estimated by itemizing costs with all values given in US dollars (Table 4.3).

4.3 Results

PDs were screened as per the methods mentioned previously and these methods were compared to those used by other FMT donor screening programs (Table 4.1).

Table 4.1 Comparison of donor exclusion criteria between different FMT research groups.

Exclusion Criteria and Tests Performed	Craven et al.	Kazerouni et al.[9]	Paramsothy et al.[10]	Costello et al.[11]	Tariq et al.[12]
History/Examination					
Between 18 and 65 years of age	X	X	X	X	X
Any medications	X		X	X	X
Antibiotics, antifungals, or antivirals in the last 3 months	X	X	X	X	X
Probiotics in the last 3 months	X		X	X	
Hospitalization in the last 3 months	X				X
Travel to high-risk areas of infectious diarrhea in the last 3 months	X	X	X	X	X
Acute diarrhea within the past 6 months					X
Tattoo or body piercing in the last 6 months	X	X	X	X	X
Known HIV or viral hepatitis exposure in the last 12 months	X	X	X	X	X
High risk sexual behaviour	X	X	X	X	X
Illicit drug use	X	X	X	X	X
Incarceration or a history of incarceration		X	X	X	X
Household members with active GI infection			X	X	
Chronic constipation					X
Any gastrointestinal disorder	X	X	X	X	X
Overweight (BMI>25)	X	X			
Obese (BMI >30)			X	X	X
Hypertension	X	X	X		X
Type 2 diabetes	X	X		X	X
Insulin sensitivity	X	X	X		X
Hyperlipidemia	X	X	X	X	X
Atherosclerosis	X	X	X		
Malnutrition (BMI<18)	X		X	X	
Autoimmune disease	X	X	X	X	X
Atopic disease		X	X	X	
Psychiatric history	X	X		X	X
Infection with HIV, Syphilis, Hepatitis B or C	X	X	X	X	X
Malignancy	X	X	X	X	X
Chronic pain syndromes, neurologic or neurodevelopmental disorders		X	X	X	X
History of major gastrointestinal surgery	X	X	X		
Any kind of liver disease	X				
Alcoholic intake >10g/day women and >20g/day men	X				
Family history of colorectal carcinoma	X	X	X	X	
Family history of diabetes	X				
Family history of early onset coronary disease, gastrointestinal or liver disease	X	X			
Stool Tests					
Ova, cysts and parasites	X	X	X	X	X
Microscopy and culture	X	X	X	X	X
Rotavirus	X	X		X	
Norovirus	X	X	X	X	

Adenovirus	X	X		X	
Clostridium difficile toxin	X	X	X	X	X
Vancomycin resistant enterococcus screen	X	X		X	X
Carbapenem-resistant *Enterobacteriaceae*	X	X			
Extended spectrum beta-lactamase producing *Enterobacteriaceae*	X	X			
Fecal *Giardia* antigen	X	X	X		X
Fecal *Cryptosporidium* antigen	X	X	X		X
Isospora		X			
Cyclospora		X			
Microsporidia	X	X			X
Blood Tests					
Complete blood count	X	X	X	X	
Electrolytes, urea and creatinine	X		X	X	
Liver function tests	X	X	X	X	
Erythrocyte sedimentation rate			X	X	
C-reactive protein		X	X	X	
Fasting lipids and blood sugar level	X			X	
Anti-tTG antibody for celiac disease	X				
Antinuclear antibody				X	
HIV type 1 and 2	X	X	X	X	X
Hepatitis A virus IgM	X	X	X	X	X
Hepatitis B virus surface antigen, Hepatitis B virus core antibody (IgM and IgG), Hepatitis B virus surface antibody	X	X	X	X	X
Hepatitis C virus antibody	X	X	X	X	X
Human T-cell lymphotropic virus 1 and 2	X	X	X	X	
Epstein Barr Virus IgM	X			X	
Cytomegalovirus IgM	X			X	
Strongyloides stercoralis, Entamoeba histolytica, Helicobacter pylori serology	X	X		X	
H. pylori	X	X		X	
Treponema pallidum screening cascade	X	X		X	
Listeria	X				
Nasal Swab					
Methicillin-resistant *Staphylococcus aureus*	X	X			
Urine Tests					
Gonorrhea and chlamydia	X				

Screening procedures of Kazerouni et al. are based on the OpenBiome safety guidelines[13].

Forty-six potential donors were screened in total (Figure 4.1) including 25 females and 21 males aged 20-73 yrs (median: 35 yrs). Of the 46 PDs that were screened, 23 passed the history and examination by a physician. The most common reasons for exclusion included having a BMI>25 and not providing a medical history. Of those 23 participants, 5 passed all blood, urine, stool and pharyngeal/rectal swab screening. The most common reasons for excluding PDs after blood and stool testing were positive tests for *Blastocystis hominis*, *Dientamoeba fragilis* and *Helicobacter pylori*. Of those five, two were subsequently excluded after they had acute gastroenteritis and another two were excluded because they travelled to tropical countries after screening. One of these potential donors also moved out of the region after screening. Therefore, after full screening of 46 potential volunteers, only 1 donor was able to donate on a regular basis for the program. The rate of acceptance for this study was similar to those found by other FMT donor screening programs (Table 4.2).

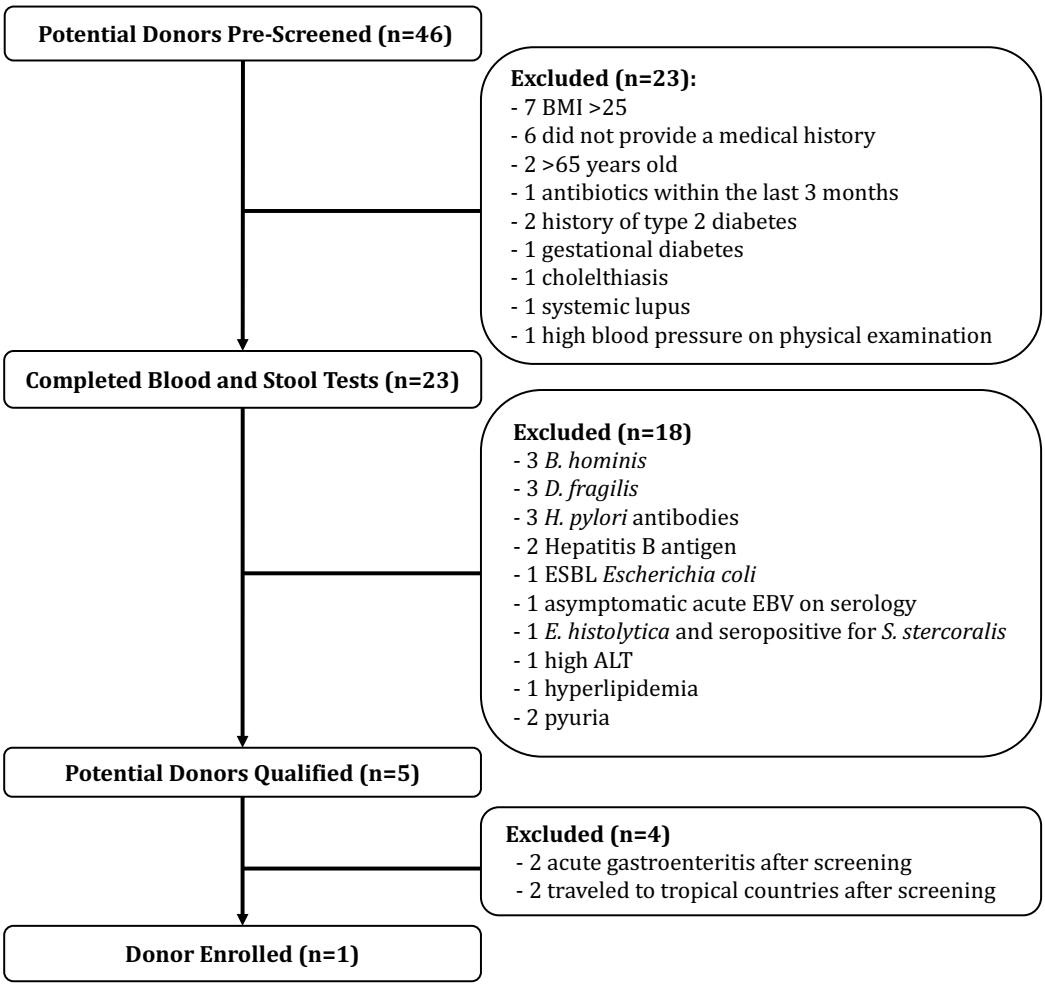

Figure 4.1 Potential donor screening outcomes using expanded donor screening methods.

Table 4.2 A comparison of various donor screening programs' acceptance rates.

Study	Sample Size	Passed History / Exam	Passed Stool Test	Passed Blood Test	Overall Acceptance
Craven et al.	46	50 % (23/46)	61 % (14/23)	57 % (13/23)	11 % (5/46)
Kazerouni et al.[9]	77	35 % (27/77)	44 % (12/27)	100% (12/12)	16 % (12/77)
Paramsothy et al.[10]	116	25 % (29/116)	48 % (14/29)	97 % (28/29)	10 % (12/116)
Costello et al.[11]	44	50 % (22/44)	68 % (15/22)	93 % (14/15)	37 % (14/44)
Tariq et al.[12]	21	43 % (9/21)	78 % (7/9)	89 % (8/9)	24 % (5/21)

The cost of a full work-up (including history, examination, blood, stool, and urine screening, and administration) at our centre was approximately $440 USD per person and those that were excluded after the history and examination cost $150 USD per person (Table 4.3). The cost of the history, examination and administration was based on the fee of a one-hour doctor's appointment and three hours of the research coordinator's time. The costs of laboratory tests performed in hospital were provided by a lab manager at St. Joseph's Health Care, London, Ontario, Canada (where the tests were performed) in October 2016. The exchange rate on June 20th, 2017 of Canadian to US dollars (0.75) was used to convert the cost of donor screening to USD. Out of the 46 donors that were screened 23 had the full work-up and 23 were excluded before full screening took place. The total for patients who had full screening was approximately $10,120 USD and the total for patients with only a doctor's visit was $3,450 USD. Eighteen PDs had abnormal laboratory results and they had a follow-up appointment with a physician adding an additional $1,620 USD in costs. To find a single donor approximately $15,190 USD was spent. If the same testing were to be completed in the United States it would cost approximately $3,770 USD per PD for laboratory tests alone (Table 4.3).

Table 4.3 Cost of screening a single donor for fecal transplant.

	Cost per person in Canada (USD)	Cost per person in US (USD)
Stool Tests		
Ova, cysts and parasites	$21.00	$138.84
Microscopy and culture	$13.50	NA†
Rotavirus	NA‡	$97.94
Norovirus	NA‡	$108.70
Adenovirus	NA‡	$78.02
Clostridium difficile toxin GDH and toxin	$30	$100.09
Vancomycin resistant enterococcus	$2.63	NA†
Fecal *Giardia* antigen	$10.88	$88.15
Fecal *Cryptosporidium* antigen	$10.88	$86.10
H. pylori	NA‡	$169.69
Extended spectrum beta-lactamase producing *Enterobacteriaceae*	NA‡	$77.49
Microsporidia	NA‡	$72.11
Blood Tests		
Complete blood count	$6.20	$42.18
Electrolytes	$7.77	$39.69
Urea	$1.94	$49.04
Creatinine	$1.94	$46.64
Alanine aminotransferase	$1.94	$19.03
Alkaline phosphatase	$1.94	$49.04
Total bilirubin	$1.94	$19.03
Albumin	$1.94	$41.16
Fasting lipids	$10.48	$147.35
HbA1c	NA‡	$71.39
Glucose	$1.94	$34.80
Anti-tTG antibody (for celiac disease)	$11.95	$156.68
HIV type 1 and 2	NA‡	$110.85
Hepatitis A virus IgM	$15.00	$96.86
Hepatitis B virus surface antigen	$17.25	$398.44
Hepatitis B virus core antibody IgM and IgG	$14.42	$107.63§
Hepatitis B virus surface antibody	$15.46	$52.28
Hepatitis C virus antibody	$15.14	$66.73
Human T-cell lymphotropic virus 1 and 2	NA‡	$110.85
Epstein Barr Virus IgM	$26.25	$75.34
Cytomegalovirus IgM	$30.00	$76.41
Strongyloides stercoralis serology	NA‡	$145.29
Entamoeba histolytic serology	NA‡	$96.86
Helicobacter pylori serology	NA‡	NA†
Treponema pallidum screening cascade	NA‡	$75.78
Listeria	NA‡	$127.00
Swabs		
Gonorrhea	NA‡	$150.68
Chlamydia	NA‡	$75.24

MRSA	$15.00	$159.60
Carbapenem-resistant *Enterobacteriaceae*	NA‡	$171.12
Total per person (laboratory testing)	**$287.39**	**$3,772.49**
Other Costs		
Administrative fee	$56.25	NA
Doctor's visit	$90.00	NA
Advertising	$4.88	NA
Total per person	**$438.52**	**NA**

NA†: not available because same test isn't offered by diagnostic service.

NA‡: not available because cost was not publicly available in Canada by the provincial health laboratory.

§: IgM only

Canadian costs include the fixed cost of advertising, supply costs of materials used, and time spent scheduling and screening donors. American costs include the supply costs of materials used and labor.

4.4 Discussion

The demand for donors for FMTs is increasing. The criteria for donor screening amongst institutions are inconsistent and recruitment of donors for FMT clinics and studies can be very difficult. Knowing the total number of people to screen to find a suitable number of donors is helpful when determining how much recruitment will need to be done. This also determines the program feasibility and costs of establishing a program. We found that of 46 volunteers screened, only 5 passed clinical and laboratory screening and due to subsequent events only 1 out of 5 was available for ongoing donation. Of the 4 donors who were successfully screened and passed all of the tests, but subsequently excluded, 2 may be available for donation at a later date after completing repeat screening as the other 2 declined repeat screening. Tariq et al.[12] had a similar experience in which almost half of their accepted donor pool were excluded after passing screening, with one donor becoming pregnant, two testing positive for Shiga toxin in stool and one opted out of being a donor.

It was found that 11% of our potential donors screened were eligible to be donors for FMTs. This 11% success rate is comparable to both Kazerouni et al.[9] and Paramsothy et al.[10] who found that 15.6% and 10.3% of potential donors passed the screening process, respectively.

OpenBiome, the longest standing international stool bank, currently reports that less than 3% of PDs applying to their program are accepted to be donors[13]. Costello et al.[11] found higher rates of success for stool tests (68%) compared to our data and that of others[8,9]. We found that the most common reasons for exclusion in our cohort were *B. hominis*, *D. fragilis* and *H. pylori.* Both *B. hominis* and *D. fragilis* were also the leading reasons for exclusion found by Paramsothy et al.[10]. While common problems, the loss of successful PDs to acute gastroenteritis and travel to tropical countries appears to be uncharacteristically high in this pool of PDs. It would be very difficult to prevent these problems from occurring in the future as we have no control over the wide variety of factors that may cause acute gastroenteritis and a donor's vacation plans. None of the PDs had plans for upcoming travel when screened, however, many were University undergraduate or graduate students and last-minute travel was a common phenomenon. If restrictions were placed on donors about where they could travel, donor retention would likely suffer.

Kazerouni et al.[9] estimated the cost of screening one donor for their public stool bank (OpenBiome) to be $885 USD per person. We estimated the cost of this screening program at $440 USD per person in Canada, with the total cost of screening to find one viable donor being $15,190 USD. The difference in costs is likely, at least partially, related to a lower cost for medical procedures, laboratory tests, and physician time in Canada than in the US. For example, the cost of laboratory test in Canada versus the United states are; ova, cysts and parasites: $21.00 USD versus $138.84 USD, *C. difficile* toxin: $30.00 USD versus $100.09 USD, and Hepatitis B virus core antibody IgM: $14.42 USD versus $107.63 USD (The pricing of Quest Diagnostics laboratory tests was used to provide costs in the US). Overall, the cost of the same laboratory testing in Canada versus the United States was approximately $290 and $3,770, respectively. There are several tests that were provided free of charge in Canada by the provincial public health laboratory (ex. Gonorrhea, chlamydia, and HTLV 1 and 2) and this was also a contributing factor as to why the price of screening a PD in Canada was significantly lower than in the US. No cost estimates were publicly available for the laboratory tests performed by the provincial public health laboratory.

The one donor successfully selected will require ongoing intermittent routine rescreening every six months. At present, there is no consensus on the frequency of rescreening, nor have they been specified in clinical guidelines[3]. Interim testing varies amongst programs from every month[11] to six months' time. Other common reasons for donor subsequent exclusion could include travel to the tropics, requiring antibiotics, or starting a new sexual relationship. This has now necessitated rescreening a large cohort of new volunteers. Three additional donors were recruited using these screening criteria before the NAFLD or MS randomized controlled trials commenced.

Finally, there is data suggesting that the efficacy of FMT for different diseases with microbiome indications varies by donor characteristics that are difficult to predict. Repeated donations from different donors were required for success in a landmark *C. difficile* therapy study[14]. Furthermore, a particular donor appeared to be especially effective in a study of FMT for ulcerative colitis[15], but whether the same donor's microbiome would also be ideal for other indications is unknown. We included a wide range of ages (18-65) in PD screening. Although some authorities note that microbiome senescence may lead to lower efficacy with an older donor, the longer a patient has lived without complications, the more likely they may have a healthy microbiome. Ideally multiple donors would be screened to find ones that are repeatedly successful for different diseases treated by FMT. This would dramatically increase the number of donors required for screening and increase costs to have a stool bank that specializes in treating multiple diseases.

Limitations of this study include that the sample size was small (n=46) and the reasons for exclusion after PDs passed screening may not be generalizable for other populations, as the rates of acute gastroenteritis and tropical travel in the 5 accepted PDs were unusually high. The cost of screening a PD for FMT is underestimated because the costs of tests from the provincial health laboratory were not made available and these tests were performed in Canada where the cost of physician's time and laboratory testing is significantly lower than in the United States. The strengths of this study are that it shows a minimum cost estimate for clinics thinking of using FMTs and establishing their own pool of donors and the

difficulties that can be anticipated to establish and maintain a donor pool from a single center without significant funding.

Our data raise the concern regarding the feasibility of individual centers establishing and maintaining FMT donor pools. Reimbursement for FMT is not high enough to counter the costs of screening PDs for programs, for example in the US an FMT is reimbursed $76 USD through Medicare. In Ontario, there is no reimbursement whatsoever. Central banks with storage and shipment of frozen samples may be necessary to maintain programs[16]. However, to enable this, full transparency and reporting of all screening protocols will be necessary to enable clinicians and regulators to determine the acceptability and potential efficacy of biobank stools for their clinical context.

Novel uses for FMTs, as well as new insights on how the microbiome affects human health, have created a demand for stricter exclusion criteria for donors. Anticipating the number of potential donors that must be screened to find a suitable donor can help to determine the amount of recruitment that must be done and the funds required to accomplish this. The large number of donors who require screening and the resultant cost may make the establishment of multiple local programs a nonviable goal with central processing and shipment being a more reasonable alternative.

4.5 References

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Chapter 5

5 Insulin resistance, percentage liver fat, and intestinal permeability of patients with non-alcoholic fatty liver disease following fecal microbiota transplantation

5.1 Introduction

Non-alcoholic fatty liver disease is an obesity-related disorder characterized by having more than 5% fat by volume in the liver. NAFLD affects 20-30% of North American adults and 80% of obese individuals[1]. The metabolic syndrome is present in 67% of NAFLD patients[2]. It has been well-established that the gut microbiome plays a role in metabolism[3-6]. Many have postulated that one of the reasons that obese individuals develop NAFLD is due to differences in the composition of bacteria in the gut. Evidence from animal studies showed that transfer of the gut microbiota from obese mice or from obese humans into germ-free mice reproduced the obese phenotype[7]. Yet, there have been numerous studies that have compared the gut microbiota of NAFLD, obese, and healthy individuals without finding consistent differences at the phylum or genus level[8-12].

As opposed to certain bacteria being responsible for the pathogenesis of NAFLD, bacterial metabolites may be the driving force. Butyrate is a short-chain fatty acid produced by the breakdown of fibre by a variety of bacteria in the gut. It increases intestinal barrier integrity and reduces the amount of LPS that passes through the intestinal membrane. A decrease in butyrate and an increase in the amount of LPS passing through the intestinal membrane has been shown in mice to cause NAFLD and insulin resistance[13]. Genetic susceptibility[14], hyperglycemia[15], and bacterial pathogens[16] can also increase gut permeability. One study found that administering butyrate to mice fed a high-fat diet increased their energy expenditure and protected them from developing insulin resistance[17]. A human study has also demonstrated that NAFLD patients have significantly increased gut permeability compared to healthy controls, and there was a correlation with greater amounts of fat in the liver[18]. Interventions to reduce gut permeability in humans have yet to be developed.

Given the relationship between the microbiome, NAFLD and the metabolic syndrome, FMT is being investigated to alter the microbiota composition of the intestine and treat some of these diseases. A study in mice demonstrated that insulin resistance and the fatty liver phenotype could be transmitted via FMT[19]. A human study that administered FMT to 18 metabolic syndrome patients (9 allogenic (from a thin donor) and 9 autologous transplants) reported a significant increase in insulin sensitivity in the allogenic transplant group (26.2 to 45.3 μmol/kg/min)[20]. Notably, the autologous transplant group did not experience a change in insulin sensitivity. The authors suggested that the improvement in insulin sensitivity was due to an increased abundance of butyrate-producing bacteria although a subsequent study did not confirm the butyrate hypothesis but questioned the impact of microbial acetate production[21].

We hypothesized that an FMT from a lean, healthy donor given to NAFLD patients with metabolic syndrome would result in a decrease in insulin resistance (the primary outcome), and small intestinal permeability (a secondary outcome) both at 6 weeks post-FMT, and hepatic PDFF at 6 months (a secondary outcome). A pilot study of twenty-one subjects was carried out to test the hypothesis.

5.2 Methods

5.2.1 Patient Recruitment and Randomization

Between June 2016 to April 2018, twenty-one NAFLD patients were recruited by hepatologists in London, ON, Canada. This was a double-blinded randomized controlled trial. Patients were randomly assigned at a ratio of 3:1 to receive an allogenic or autologous FMT and had follow-up appointments for 6 months' post-transplant. A summary of the timeline of appointments and tests performed can be found in Supplementary Table 5.1.

5.2.2 Sample Size Calculation:

Using the individual patient data from Vrieze et al.[20], the mean (standard deviation) rate of glucose disappearance for patients receiving allogenic FMT infusion was estimated to be 30.7 (15.3) at baseline and 38.1 (19.2) at six-week follow-up. The correlation between time

points was 0.91. The sample size calculation for paired sample t-test, using a two-sided test, power of 80%, and alpha of 0.05, yielded a minimum sample size of 12 allogenic patients.

5.2.3 Patient Inclusion Criteria

- Attendance at the gastroenterology/hepatology clinic with a diagnosis of NAFLD as per the AASLD criteria[22].
- Willingness to provide informed Consent.
- > 18 yrs old

5.2.4 Patient Exclusion Criteria

- Type 1 or 2 diabetes requiring insulin (oral hypoglycemics were not excluded as long as there was no change in dosage for at least three months and no plan to adjust the dose).
- Inability to attend follow-up visits.
- Inability to provide informed written consent.
- Ongoing use of antibiotics or probiotics.
- Previous or planned bariatric surgery.
- Presence of a chronic intestinal disease e.g. celiac disease, malabsorption, or colonic tumor.
- Immunosuppression from transplantation, HIV, cancer chemotherapy or ongoing use of any immune-suppressive agents.
- Pregnancy

5.2.5 Donor Selection

Forty-six potential donors were screened to find three suitable donors for this study. The methods of selection have been described elsewhere[23]. Potential donors were excluded if there was any history in the patient or immediate family (i.e. parents, siblings or children) of metabolic disease (i.e. hypertension, hyperlipidemia, diabetes, or obesity), vascular, liver, autoimmune, or psychiatric disease. Patients with a history of, high-risk activities to

acquire infectious agents (new sexual partner, hospitalization or travel to the tropics within 3 months) or antibiotic therapy within 3 months were excluded. Potential donors underwent a full physical examination as well laboratory testing to rule out body mass index (BMI) $\geq$25 kg/m^2, hypertension, hyperlipidemia, elevated transaminases or glycated hemoglobin (HbA1c) prior to donation. If they passed the medical examination, their stool, blood and urine were tested for 30 different bacterial, viral and protozoan agents to ensure that known transmissible diseases would not be passed along to recipients through FMT. Only 1 in 10 potential donors qualified for this study. Three donors in total were identified and all provided fresh stool for the allogenic transplants. Donor characteristics are summarized in Supplementary Table 5.2.

5.2.6 Fibrosis Staging

Liver fibrosis stage was diagnosed using a variety of methods as various clinicians were involved with recruitment for this study. Biopsy (n=9), FibroScan (n=7), and MR elastrography (n=5) were used. All patients had hepatic steatosis documented by ultrasound. Liver biopsies were analyzed by experienced hepatopathologists and fibrosis staged using the Brunt methodology[24] (F0 = no fibrosis; F1 = zone 3 pericellular/sinusoidal fibrosis, focal or extensive; F2 = zone 3 plus focal or extensive periportal fibrosis; F3 = bridging fibrosis, focal or extensive; and F4 = cirrhosis). The method of histologic scoring used was the Non-Alcoholic Fatty Liver Disease Activity Score (NAS), a validated scoring system for the evaluation of histologic changes in NAFLD[25]. For FibroScan, fibrosis stage was determined according to that established by Wong et al. (F0-1 $\leq$ 7.0 kPa, F2 7.1 – 8.6 kPa, F3 8.7 - 10.3 kPa, F4 $\geq$ 10.4 kPa)[26]. For MR elastography, fibrosis stage was determined according to that established by Loomba et al.[27].

5.2.7 Fecal Microbiota Transplant

All patients were asked to drop off a fresh fecal sample within 72 hours of their scheduled FMT to keep them blinded as to whether they were going to receive an allogenic or autologous FMT. Whole stool was stored at 4 °C and processed immediately prior to transplantation. All patients were pretreated with a bowel cleanse using 3

envelopes/sachets of Pico Sulphate preparation immediately after donating a baseline stool sample and prior to the FMT. To prepare the FMT material, 2 g of stool (from either the donor or autologous sample) and 125 mL of sterile saline were placed inside of a BA614/STR filter bag (Seward, Islandia, NY) and mixed using the Stomacher® 400 Circulator (Seward, Islandia, NY) at 230 rpm for 30 seconds. The filtered material was then transferred into sterile sample collection containers, transferred to the endoscopy unit and used within 2 hours. The FMT (allogenic or autologous) was delivered to the duodenum using an endoscope. Dr. Adam Rahman performed the FMT delivery under general anesthesia at University Hospital, London, ON.

5.2.8 Small Intestinal Permeability

Patients were asked to drink a solution of 5 g of lactulose (Calbiochem®, EMD Millipore Corp., Billerica, MA), 2 g of mannitol powder (BDH®, VWR analytical, Mississauga, ON), 1.5 g of Kool Aid (Kraft Foods, Ingleside, ON), 100 g of sucrose, and 450 mL of tap water the evening before their baseline and 6 weeks appointments. The subjects were asked to collect all the urine that they passed throughout the night and morning of their appointment and store it in a urine collection bottle. This bottle was brought to the clinic, the total volume of urine was recorded and then aliquoted into 10 mL amounts. Concentrations of lactulose, mannitol and sucrose were determined using high performance liquid chromatography[28]. Urine samples were sent to the lab of Dr. Jon Meddings in Calgary, AB, at the end of the study to be analyzed.

5.2.9 Blood Samples

Blood was collected from patients (fasting) at baseline, 2 weeks, 6 weeks and 6 months post-FMT. The blood was used to examine: CBC, albumin, bilirubin, glucose, fasting insulin, HbA1c, non-esterified fatty acids, cholesterol, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, total: HDL cholesterol ratio, triglycerides, apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (Alk Phos). Baseline testing for hepatic fibrosis was performed by liver biopsy and hepatic

Magnetic Resonance Elastography. Blood samples were collected by Ms. Mala Ramu at the Centre for Clinical Investigations and Therapeutics at University Hospital, London, ON.

5.2.10 Fecal Sample Collection

The fecal samples were collected to assess changes in the fecal microbiota composition following FMT using a previously validated protocol[29]. Briefly, patients collected a visibly soiled piece of toilet paper after passing a stool at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-transplant. The subjects placed the fecal sample in a Fisherbrand™ Opaque Sterile Sampling Bag (Fischer Scientific, ThermoFisher Scientific, Mississauga, ON) and brought it to their appointments. The samples were then frozen at -80 °C until DNA extraction took place.

5.2.11 DNA Extraction

DNA from the toilet paper samples was extracted using the DNeasy® Powersoil® HTP 96 Kit (Qiagen, Toronto, Ontario, Canada), as per the manufacturer's instructions with the following modification: A centrifuge speed of 3700 rpm for 10 minutes was used. Extracted DNA was stored at -20 °C until amplification.

5.2.12 DNA Amplification

The BioMek® 3000 Laboratory Automation Workstation for automated PCR reagent set up was used to load 10 µL (2.3 pmol/µL) of 32 primers (16 left and 16 right) with unique barcodes into 96 well plates. Amplifications of the V4 region of the 16S ribosomal RNA gene were carried out with the primers (5′-3′) ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNxxxxxxxxGTGCCAGCMG CCGCGGTAA and (5′-3′) CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

NNNNxxxxxxxxGGACTACHVGGGTWTCTAAT (xxxxxxxx is a sample specific nucleotide barcode and the preceding sequence is a portion of the Illumina adapter sequence for library construction). The BioMek® robot was then used to transfer 2 µL of

template DNA into the primer containing 96 well plates. Then 20 µl of Promega GoTaq® Colourless Master Mix (Promega, Maddison, WI) was added to the DNA template and primers. The final plate was firmly sealed with a foil PCR plate cover. This plate was placed in the Eppendorf Mastercycler® thermal cycler (Eppendorf, Mississauga, Ontario, Canada), where the lid was kept at 105 °C. An initial warm-up temperature of 95 °C was used for 2 min to activate the GoTaq®. Afterwards, the volumes underwent 25 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. After completion, the temperature of the thermal cycler was held at 4 °C, and amplicons were then stored at − 20 °C.

5.2.13 DNA Sequencing and Data Analysis

Amplified DNA was sent to the London Regional Genomics Centre at Robarts Research Institute (Western University, London, Ontario, Canada). The samples were quantified (Quant-it, Life Technologies, Burlington, Ontario, Canada) and pooled at equimolar concentrations. The pooled libraries were cleaned using QIAquick (Qiagen, Germantown, Maryland, USA) and then sequenced using the MiSeq Illumina® platform, with 2 × 300 bp paired-end chemistry. The reads were demultiplexed and filtered using dada2 (version 1.8) and custom R scripts written by Greg Gloor (github.com/ggloor/miseq_bin). The demultiplexed reads will be available at NCBI SRA. Any taxa with less than 3 counts in 30% of the samples were removed. Taxonomy was assigned using an RDP classifier provided by the dada2 package and trained against version 132 of the SILVA database. Diversity of the fecal microbiota was quantified based on Shannon's index and was calculated using the Vegan package (github.com/vegandevs/vegan). ALDEx2 was used to identify differentially abundant taxa between patients with abnormal permeability and normal permeability as well as patients with abnormal permeability at baseline and 6 weeks following FMT[30]. ALDEx2 was also used to identify differentially abundant taxa between baseline and all subsequent time points for allogenic and autologous FMT recipients. An effect size cutoff of > |3| was used.

5.2.14 Magnetic Resonance Imaging (MRI)

Patients underwent an abdominal MRI (3T), including 3D chemical shift encoded MRI[31], at their baseline and 6-month appointment (conducted at the Robarts Research Institute). MRI data were analyzed to determine abdominal total volume (cm^3), abdominal subcutaneous adipose volume (cm^3), abdominal visceral volume (cm^3), and liver proton density fat fraction (PDFF, %)[32]. Two patients were unable to have an MRI; one had a pacemaker and one was unable to fit into the apparatus. MRIs were received by Dr. Charles McKenzie at University Hospital, London, ON.

5.2.15 Diet History Questionnaire

Prior to their FMT and six weeks after their FMT, participants completed the online diet history questionnaire version 2 (DHQII) with portion sizes, which measured intake over the previous month. Downloaded nutrient data were reviewed by the study dietitian for plausibility: e.g. whether energy and nutrient intakes were likely to be physiologically possible and if appropriate this was checked with participants. Under-reporters were included in the analysis. Due to inaccuracies in estimation, all dietary data are reported to a maximum of two significant figures. Students' t-tests were used to compare differences in changes in intakes. The diet history questionnaire was collected and analyzed by Dr. Ruth Harvie at St. Joseph's Health Care, London, ON.

5.3 Results

5.3.1 Insulin resistance and PDFF were not improved with allogenic FMT

Patients were randomly assigned to either the allogenic (n=15) or autologous (n=6) FMT group. By chance, patients who were randomized to the autologous FMT group had less severe disease scoring and healthier levels of a variety of biochemical markers (Table 5.1). Histological scoring of NAFLD patients can be found in Supplementary Table 5.3.

Table 5.1 Characterization of patients at baseline.

Variable	Allogenic FMT (n=15)	Autologous FMT (n=6)	Normal Range
Age (years)	47.6 (14.9)	57.5 (13.0)	
Sex (Female:male)	10:5	5:1	
Height (cm)	168.7 (10.2)	169.1 (7.4)	
Weight (kg)	103.6 (18.0)	107.6 (31.4)	
Waist to Hip Ratio	0.962 (0.053)	0.961 (0.048)	
BMI	36.3 (5.0)	37.4 (9.5)	18.5-25
Chemistry			
Albumin (g/L)	44.4 (2.0)	42.5 (2.3)	35-50
Bilirubin (µmol/L)	8.4 (2.0)	8.7 (4.6)	<20.5
Glucose, fasting (mmol/L)	7.3 (1.8)	7.9 (2.8)	3.5-5.8
Insulin (pmol/L)	196 (177)	166 (129)	<174
HOMA-IR	3.5 (1.3)	4.4 (2.1)	<1.7
HbA1c (%)	6.3 (0.9)	6.4 (1.0)	4-6
Lipids			
Total cholesterol (mmol/L)	4.68 (1.15)	3.53 (1.08)	<5.2
HDL cholesterol (mmol/L)	1.04 (0.25)	1.18 (0.25)	>0.9
LDL cholesterol (mmol/L)	2.68 (1.09)	1.76 (0.84)	<2.0
Total: HDL cholesterol ratio	4.8 (1.6)	3.0 (0.5)	<5.0
Triglycerides (mmol/L)	2.30 (1.43)	1.31 (0.25)	1.7
Non-esterified fatty acids (µmol/L)	562 (238)	616 (269)	720
Apo A1 (g/L)	1.59 (0.26)	1.69 (0.23)	
Apo B (g/L)	1.13 (0.35)	0.78 (0.22)	
ApoB:ApoA1 ratio	0.72 (0.21)	0.46 (0.10)	
Liver Enzymes			
Alanine Aminotransferase (U/L)	59 (27)	37 (7)	17-63
Aspartate Aminotransferase (U/L)	38 (23)	31 (5)	14-40

Alkaline Phosphatase (IU/L)	71 (19)	74 (16)	38-126
Abdominal Total Volume (cm^3)	17945 (4414)	10747 (6914)	
Abdominal Subcutaneous Adipose Volume (cm^3)	11911 (3951)	8021 (3277)	
Abdominal Visceral Fat Volume (cm^3)	6041 (1878)	6776 (877)	
Hepatic PDFF (%)	19.24 (8.33)	23.87 (14.56)	<5
Liver Fibrosis Scoring			
F0	4	3	
F1	5	1	
F2	2	0	
F3	1	0	
F4	4	2	

Data presented are the mean (SD) of patients at baseline.
Normal Ranges are defined by the Medical Council of Canada[33].

The primary outcome for this study was insulin resistance, as measured by the HOMA-IR score. Wilcoxon matched-pairs signed rank test was used to compare HOMA-IR at baseline and 6 weeks post-FMT in both the allogenic and autologous groups. There was no significant decrease in the insulin resistance of patients who received an allogenic or autologous FMT (Figure 5.1a and b). One patient in the allogenic group had specific insulin and fasting glucose concentrations above the limit used to calculate the HOMA-IR score on both baseline and 6-week assessments. This patient was not included in the HOMA-IR analysis.

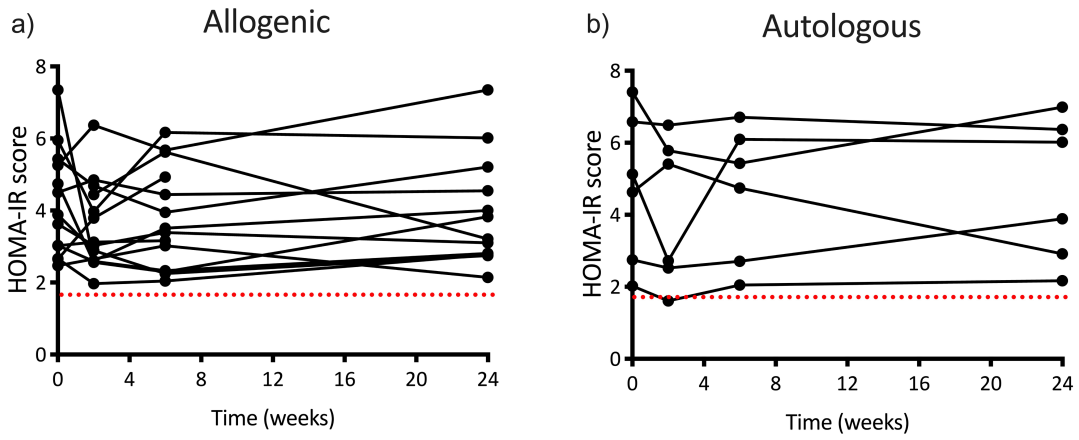

Figure 5.1 Insulin resistance was not significantly altered by allogenic nor autologous FMT. HOMA-IR score was calculated using fasting glucose and insulin (specific) concentrations. Wilcoxon matched-pairs signed rank test was performed to compare the HOMA-IR scores at baseline and 6 weeks post-FMT in both the allogenic and autologous groups (p=0.216 and p=0.688, respectively). A) Individual changes in insulin resistance in patients receiving an allogenic FMT (n=14). Median (IQR); baseline: 3.88 (2.840-5.345), 2 weeks: 3.12 (2.56-4.57), 6 weeks: 3.45 (2.32-5.10), and 6 months: 3.52 (2.78-5.05). B) Individual changes in insulin resistance in patients receiving an autologous FMT (n=6). Median (IQR); baseline: 4.88 (2.57-6.79), 2 weeks: 4.07 (2.29-5.96), 6 weeks: 5.09 (2.55-6.25), and 6 months: 4.96 (2.73-6.53 HOMA-IR). HOMA-IR: homeostatic model assessment – insulin resistance. The dotted line represents the cutoff for a normal HOMA-IR score, <1.7.

There was no significant difference in the hepatic PDFF 6 months' post-transplant in patients who received an allogenic or an autologous FMT (Figure 5.2a and b). Wilcoxon matched-pairs signed rank test was used to compare the hepatic PDFF at baseline and 6 months' post-FMT in both the allogenic and autologous groups. Two patients in the autologous group were unfit to have an MRI. A detailed report of each patient's NAS can be found in Supplementary Table 5.3.

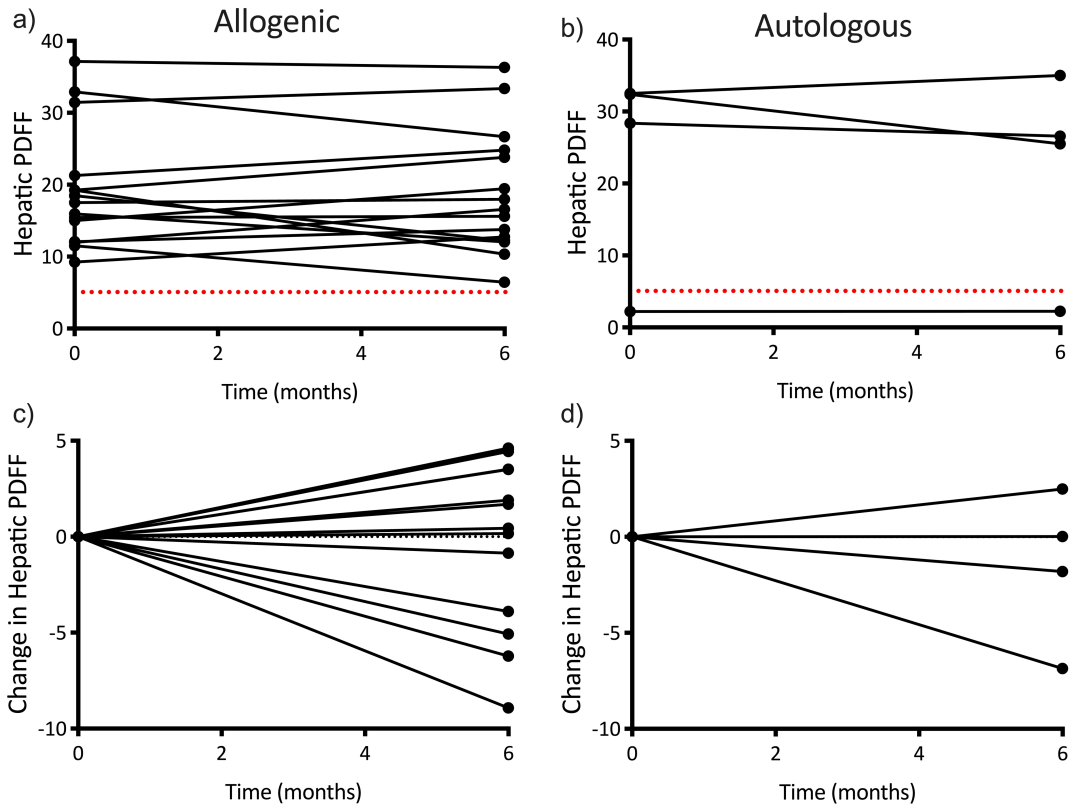

Figure 5.2 The hepatic PDFF in NAFLD patients was not significantly altered by FMT. The hepatic PDFF was determined by MRI. Wilcoxon matched-pairs signed rank test was performed to compare the hepatic PDFF at baseline and 6 months' post-FMT in both the allogenic and autologous groups (p=0.804 and p=0.875, respectively). A) The hepatic PDFF over time in patients who received an allogenic FMT (n=15). Median (IQR); baseline: 17.52 % (12.1-21.29 %) and 6 months: 16.6% (12.26-24.8 %). B) The hepatic PDFF over time in patients who received an autologous FMT (n=4). Median (IQR); baseline 30.38% (8.759-32.48 %) and 6 months: 25.52 % (8.06-32.89 %). C) The change in the hepatic PDFF in patients who received an allogenic FMT (n=15). D) The change in the hepatic PDFF in patients who received an autologous FMT (n=4). The dotted line represents the cutoff for a normal hepatic PDFF, < 5%.

5.3.2 Abnormal small intestinal permeability improved following allogenic FMT

Small intestine permeability was assessed using the lactulose:mannitol urine test. Seven patients in the allogenic FMT group had elevated small intestinal permeability prior to FMT. Five received an FMT from donor 1, one from donor 2, and one from donor 3.After the allogenic FMT from a lean, healthy donor, all seven patients had a decrease in their small intestinal permeability (Figure 5.3a), with two decreasing to within the normal range of permeability (defined by test values less than 0.025). There was no relationship between abnormal small intestinal permeability and fibrosis score as determined by the Kruskal-Wallis test (p=0.7767) (Supplementary Figure 5.1). There was no association of specific donors with an improvement in intestinal permeability. All three donors were successful at lowering small intestinal permeability in patients that had abnormal permeability. Elevated baseline small intestinal permeability was observed in one of patients who received an autologous FMT and it improved to within the normal range of permeability, however one patient in the autologous group had a normal baseline permeability which rose above normal at 6 weeks (Figure 5.3b). Overall there was a significant improvement in small intestinal permeability in the allogenic group (p=0.018), but not in the autologous group (p=0.563) (Figure 5.3).

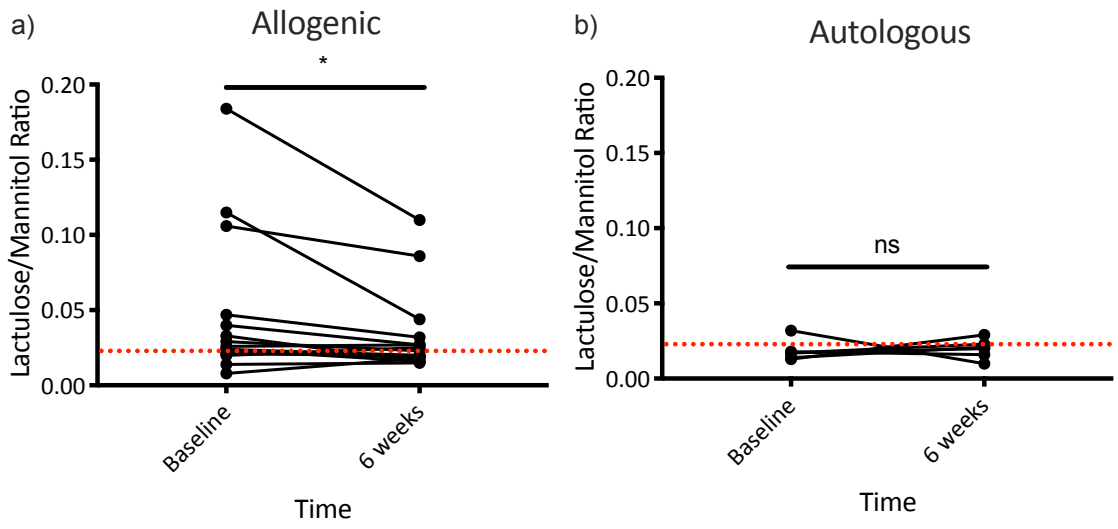

Figure 5.3 Patients who received an allogenic FMT from a thin and healthy donor showed improved intestinal permeability. Small intestine permeability was calculated using the lactulose:mannitol urine test. Ratios were non-zero and Wilcoxon matched-pairs signed rank test was performed to compare the lactulose:mannitol ratio at baseline and 6 weeks post-FMT in both the allogenic and autologous groups (*p=0.018 and p=0.563, respectively). A) Lactulose:mannitol ratio of patients who received an allogenic FMT (n=15). Median (IQR); baseline: 0.026 (0.021-0.047) and 6 weeks: 0.023 (0.018-0.032). B) Lactulose:mannitol ratio of patients who received an autologous FMT (n=6). Median (IQR); baseline: 0.017 (0.0138-0.0215) and 6 weeks: 0.0205 (0.0145-0.0245). The dotted line represents the cutoff for the normal lactulose:mannitol ratio, <0.025[35].

5.3.3 Fecal microbiota diversity did not relate to metabolic or intestinal permeability responders

A recent study of patients with metabolic syndrome suggested that peripheral insulin sensitivity improved (≥10%) only in the subset of allogenic transplants that had reduced fecal microbial diversity at baseline (although the FMT did not change the fecal microbial diversity in either group)[21]. Allogenic insulin sensitivity responders (≥10% improvement in HOMA-IR at 6 weeks) and non-responders were compared and there was no difference

in baseline fecal diversity between the two groups, although paradoxically the diversity did increase post-FMT in the non-responder group (Figure 5.4).

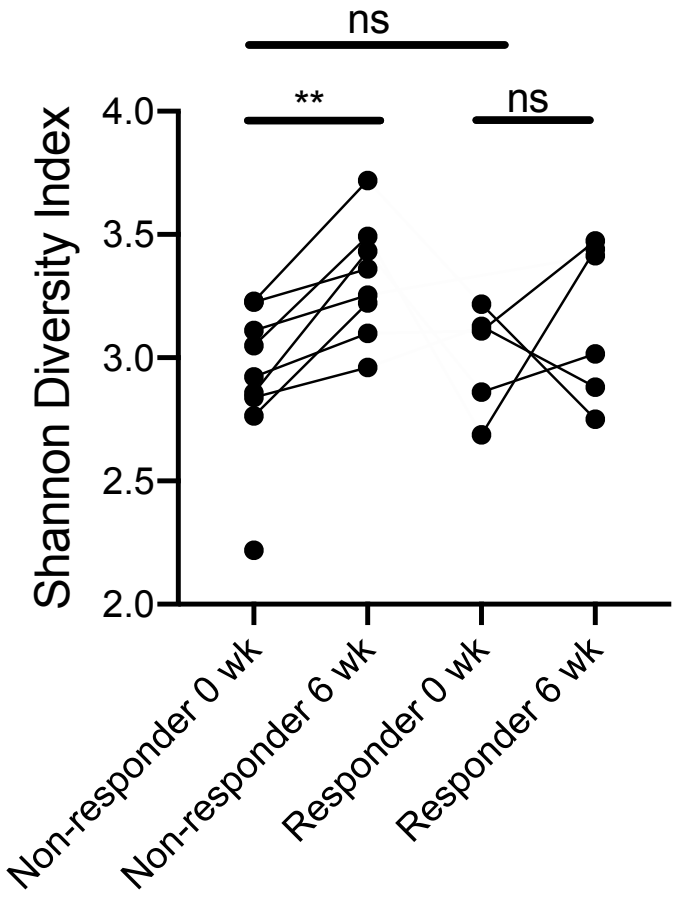

Figure 5.4 Fecal bacterial diversity is not related to metabolic response. Shannon Diversity Index at baseline and 6 weeks' post allogenic FMT is shown. A responder was characterized by having >10% reduction in HOMA-IR, a non-responder was characterized by having <10% reduction in HOMA-IR. Mann-Whitney t-test was used to compare the Shannon diversity at baseline in the responder (n=6) and non-responder groups (n=9) (p=0.797). Wilcoxon matched-pairs signed rank test was used to compare Shannon Diversity Index at baseline and 6 weeks' post-FMT in the non-responder and responder groups (p=0.0078, p=0.813, respectively). One patient in the non-responder group did not collect a 6 week sample.

Additionally, fecal microbiota diversity of allogenic intestinal permeability responders (defined as a patient that experienced an improvement in small intestinal permeability and had a baseline >0.025 lactulose:mannitol) and other allogenic patients (defined as a patient that did not initially have elevated small intestinal permeability; baseline <0.025 lactulose:mannitol) were compared. There was no difference in baseline fecal microbial diversity between the two groups, although intestinal permeability responders experienced an increase in fecal microbial diversity that approached significance 6 weeks post-FMT ($p=0.063$) (Figure 5.5).

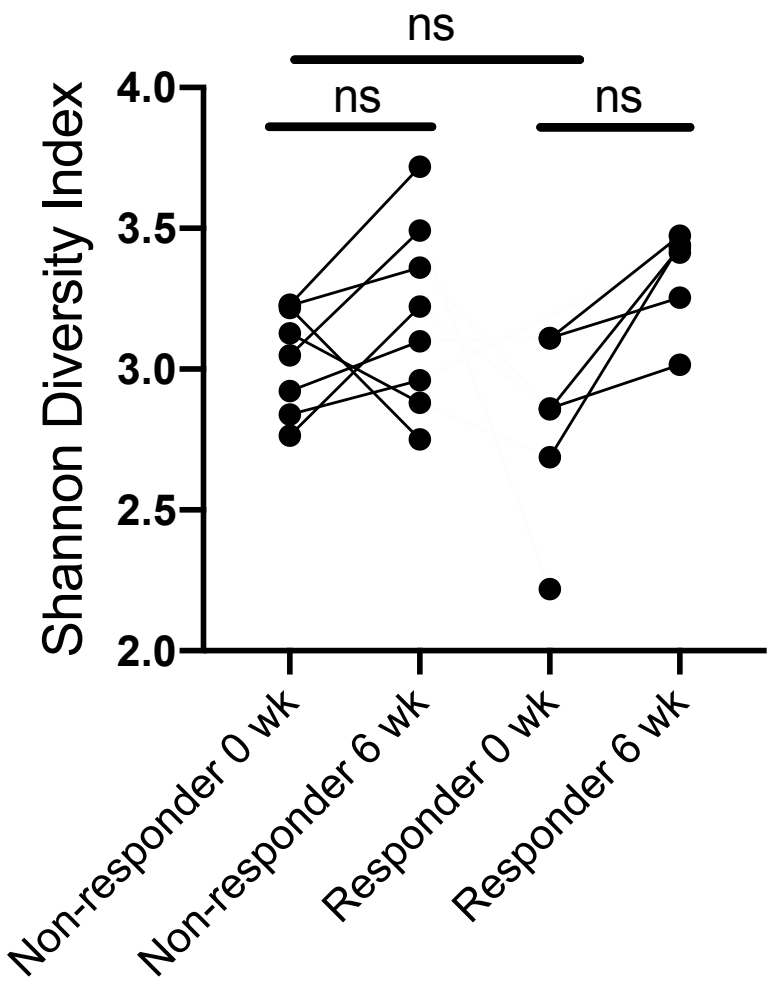

Figure 5.5 Fecal bacterial diversity and improvement in small intestinal permeability. Shannon Diversity Index at baseline and 6 weeks' post allogenic FMT is shown. A responder was characterized by having an initial lactulose:mannitol reading above 0.025 and having a reduction in this ratio at 6 weeks' post allogenic FMT. Mann-Whitney t-test was used to compare the Shannon Diversity Index at baseline in the responder (n=7) and non-responder groups (n=8) (p=0.142). Wilcoxon matched-pairs signed rank test was used to compare Shannon Diversity Index at baseline and 6 weeks' post-FMT in the non-responder and responder groups with a trend towards increasing diversity seen in the responder group (p=0.383, p=0.063, respectively). One patient in the responder group did not collect a 6 week sample.

ALDEx2 was used to identify if any particular taxa were differentially abundant in patients with abnormal permeability (>0.025 lactulose:mannitol) compared to those with normal permeability and did not find any significant differences (an effect size cut-off of >|3| was used) (Supplementary Figure 5.2). We also compared the composition of fecal bacteria in patients with abnormal permeability at baseline and 6 weeks following FMT and did not find any differentially abundant taxa (Supplementary Figures 5.3 and 5.4).

5.3.4 Changes in the fecal microbiota of FMT recipients

The fecal microbiota composition of Donor 1 and allogenic and autologous FMT recipients following transplant were examined. These changes were variable by individual in both the allogenic and autologous group (Figures 5.6 and 5.7, respectively). Detailed per subject analysis was also conducted on Donor 1 and all patients that received an allogenic or autologous transplant (Supplementary Figures 5.5-5.24). Two patients did not collect fecal samples at baseline and were excluded in this analysis; one patient from the allogenic group and one patient from the autologous group. Donors 2 and 3 moved away during the RCT and fecal samples for microbiota analysis were not available from them.

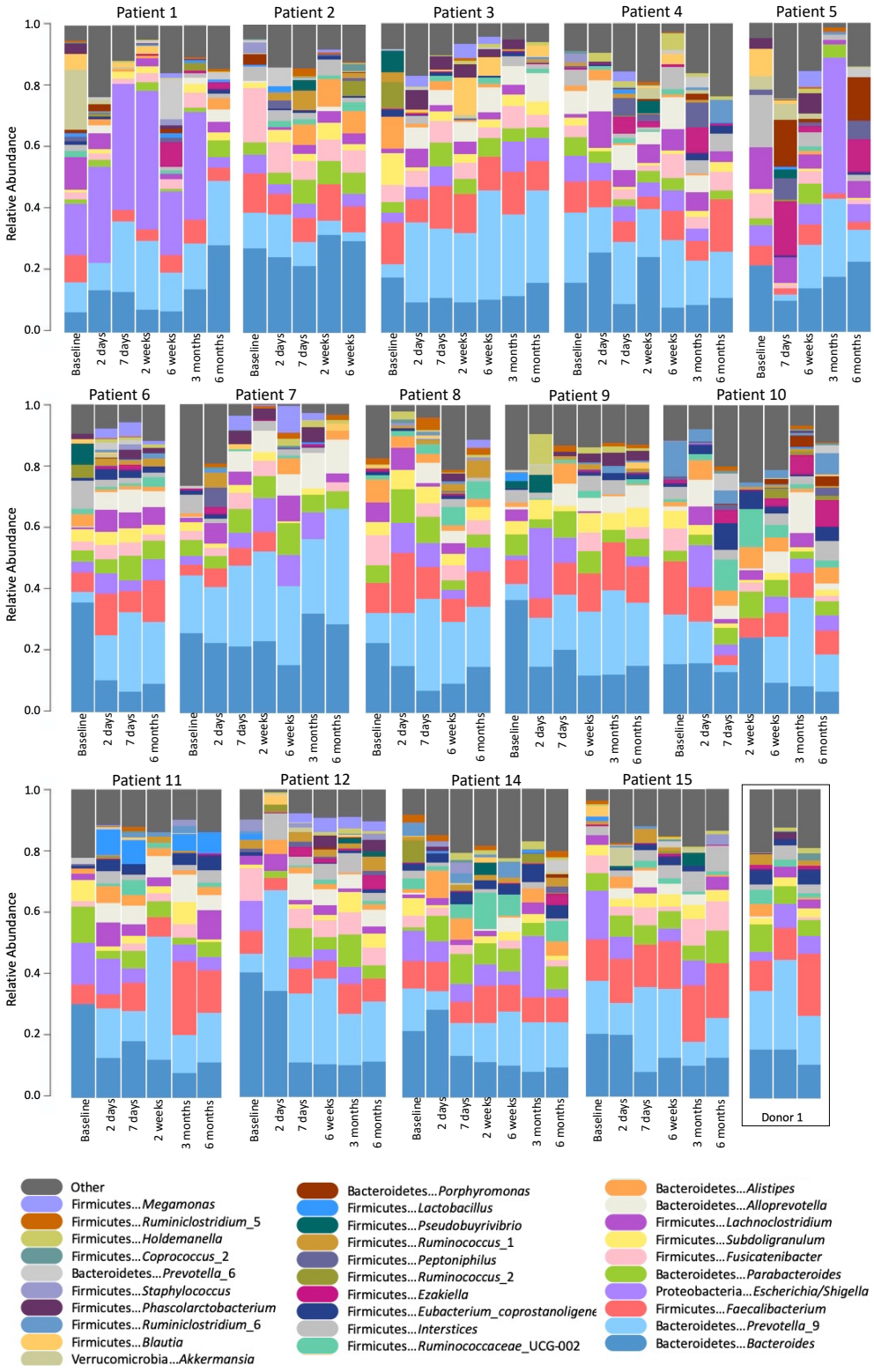
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Patient 8
Patient 9
Patient 10
Patient 11
Patient 12
Patient 14
Patient 15
Donor 1
Relative Abundance
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
2 days
7 days
2 weeks
6 weeks
3 months
6 months
Other
Firmicutes...Megamonas
Firmicutes...Ruminiclostridium_5
Firmicutes...Holdemanella
Firmicutes...Coprococcus_2
Bacteroidetes...Prevotella_6
Firmicutes...Staphylococcus
Firmicutes...Phascolarctobacterium
Firmicutes...Ruminiclostridium_6
Firmicutes...Blautia
Verrucomicrobia...Akkermansia
Bacteroidetes...Porphyromonas
Firmicutes...Lactobacillus
Firmicutes...Pseudobuyrivibrio
Firmicutes...Ruminococcus_1
Firmicutes...Peptoniphilus
Firmicutes...Ruminococcus_2
Firmicutes...Ezakiella
Firmicutes...Eubacterium_coprostanoligene
Firmicutes...Interstices
Firmicutes...Ruminococcaceae_UCG-002
Bacteroidetes...Alistipes
Bacteroidetes...Alloprevotella
Firmicutes...Lachnoclostridium
Firmicutes...Subdoligranulum
Firmicutes...Fusicatenibacter
Bacteroidetes...Parabacteroides
Proteobacteria...Escherichia/Shigella
Firmicutes...Faecalibacterium
Bacteroidetes...Prevotella_9
Bacteroidetes...Bacteroides

Figure 5.6 Fecal microbiota composition of allogenic FMT transplant recipients over six months. All patients except for patients 2, 10, and 11 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.

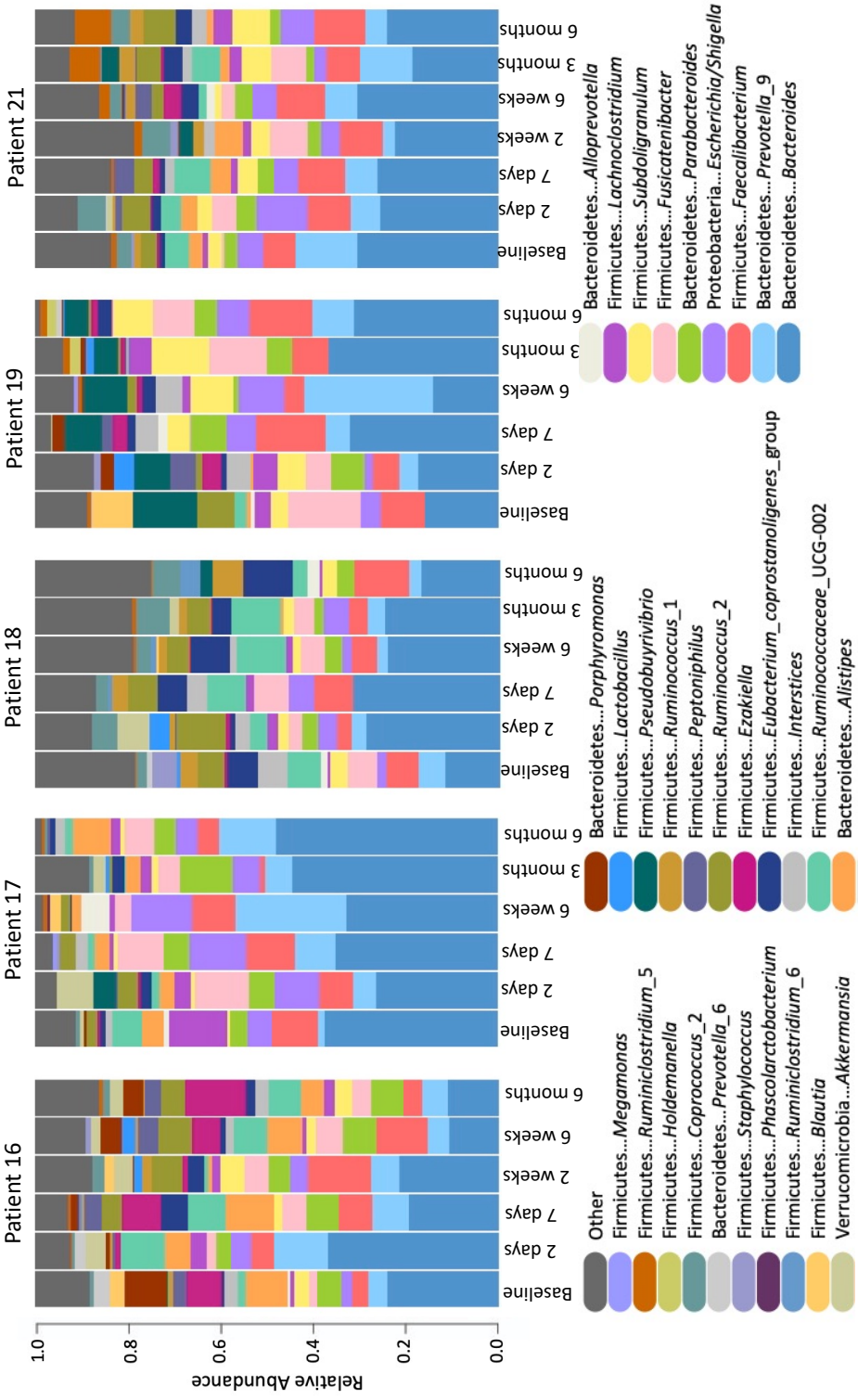
Patient 16
Patient 17
Patient 18
Patient 19
Patient 21
Baseline
2 days
7 days
2 weeks
6 weeks
3 months
6 months
Relative Abundance
1.0
0.8
0.6
0.4
0.2
0.0
Other
Firmicutes...Megamonas
Firmicutes...Ruminiclostridium_5
Firmicutes...Holdemanella
Firmicutes...Coprococcus_2
Bacteroidetes...Prevotella_6
Firmicutes...Staphylococcus
Firmicutes...Phascolarctobacterium
Firmicutes...Ruminiclostridium_6
Firmicutes...Blautia
Verrucomicrobia...Akkermansia
Bacteroidetes...Porphyromonas
Firmicutes...Lactobacillus
Firmicutes...Pseudobuyrivibrio
Firmicutes...Ruminococcus_1
Firmicutes...Peptoniphilus
Firmicutes...Ruminococcus_2
Firmicutes...Ezakiella
Firmicutes...Eubacterium_coprostanoligenes_group
Firmicutes...Interstices
Firmicutes...Ruminococcaceae_UCG-002
Bacteroidetes...Alistipes
Bacteroidetes...Alloprevotella
Firmicutes...Lachnoclostridium
Firmicutes...Subdoligranulum
Firmicutes...Fusicatenibacter
Bacteroidetes...Parabacteroides
Proteobacteria...Escherichia/Shigella
Firmicutes...Faecalibacterium
Bacteroidetes...Prevotella_9
Bacteroidetes...Bacteroides

Figure 5.7 Fecal microbiota composition of autologous FMT transplant recipients over six months. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.

5.3.5 FMT did not alter lipid profiles with the exception of NEFA

Several metabolic biochemical markers were examined in blood samples collected from the patients at each clinical visit. Exploratory analysis suggested that compared to baseline, patients 6 weeks post-allogenic FMT had lower concentrations of non-esterified fatty acids (NEFA) (mean decrease of 146.3 μmol/L; Supplementary Figure 5.25a). This was not observed in those receiving autologous FMT, Supplementary Figure 5.25b). Patients who received the allogenic FMT had a higher total:HDL cholesterol ratio than patients assigned to the autologous FMT group at baseline. A decrease in the total:HDL cholesterol ratio was observed at 6 months post-FMT in the group of patients receiving an allogenic FMT (mean decrease of 0.674; Supplementary Figure 5.26a).

No changes were observed in total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides between baseline and 6 weeks post-FMT in both the allogenic and autologous groups. (Supplementary Figures 5.27-5.30). No changes were observed in the concentration of fasting glucose over time in both the allogenic and autologous groups (Supplementary Figure 5.31). No changes were observed in the ratio of ApoB:ApoA1 between baseline and 6 weeks post-FMT in both the allogenic and autologous groups (Supplementary Figure 5.32). Weight, waist-to-hip ratio, and BMI were measured and calculated at each clinical visit (baseline, 2 weeks, 6 weeks, and 6 months post-FMT). Patients did not experience a change in their weight, waist-to-hip ratio, or BMI following an allogenic or an autologous FMT (Supplementary Figures 5.33-5.35). Seventeen patients completed the online Diet History Questionnaire Version 2 (DHQII) at baseline and at 6

weeks post-FMT (11 allogenic and 6 autologous). No changes in patients' caloric or nutrient intakes were detected (Supplementary Figure 5.36).

5.4 Discussion

To our knowledge, this is the first reported clinical trial of FMT in NAFLD patients. These data demonstrated that FMT from lean healthy donors failed to improve insulin sensitivity (Figure 5.1), the primary outcome. FMT also did not improve the percentage of fat in the liver on MRI at 6 months (Figure 5.2), a secondary outcome, but did improve intestinal permeability at 6 weeks (Figure 5.3).

The lack of improvement of insulin sensitivity stands in contrast to the findings of Vrieze et al.[20] in metabolic syndrome patients where insulin sensitivity improved with allogenic transplants (n=9 allogenic transplants). The present study of 15 allogenic patients did not demonstrate this finding; this discrepancy could be due to either technical or biological factors. Technical factors of relevance include the metric of insulin sensitivity and the study sample size. Past studies used an insulin clamp method to identify insulin sensitivity whereas this study used a HOMA-IR score for the same purpose[20,21]. Insulin clamp technology is not widely available, including at any site in Ontario. The HOMA-IR score has been shown to have excellent correlation with the insulin clamp technique[34]. Furthermore HOMA-IR is reflective of both hepatic insulin sensitivity and peripheral insulin sensitivity[34]. Past studies noted an improvement in peripheral insulin sensitivity ($p<0.05$) that was modest and did not persist, but not in hepatic insulin sensitivity[20,21]. In contrast, the present study had a larger number of patients receiving allogenic FMT than in the original study by Vireze et al.· and a smaller number of patients receiving autologous FMT[20]. While the small number of autologous samples likely did not provide sufficient statistical power to detect changes in the autologous group, our primary hypothesis (improved insulin sensitivity in allogenic FMT patients) relied solely on baseline vs. 6-week comparison within the allogenic group and therefore sample size likely does not explain the discrepancy between this study and that of Vrieze et al.[20]. Changes in HOMA-IR were not reported in previous studies[20,21]. It is possible that a small improvement in

peripheral but not hepatic insulin sensitivity may not have been adequately reflected in the HOMA-IR measure. The lack of change in the HOMA-IR raises a concern regarding the clinical impact of the small metabolic change in peripheral Insulin sensitivity seen previously.

The improvement in small intestinal permeability associated with allogenic FMT was encouraging and this is the first study to show an improvement in small intestinal permeability in NAFLD and metabolic syndrome patients following FMT. A strength of this study was the use of the gold standard lactulose/mannitol ratio as the measure of intestinal permeability. Mannitol absorption is proportional to small bowel intestinal surface area (with low values seen in conditions such as celiac disease) and lactulose has a molecular weight which normally prevents significant absorption[28]. Therefore, an increase in the ratio of urinary excretion reflects increased intestinal permeability to large molecules. It has been hypothesized that increased gut permeability is a central mechanism of gut microbiome related autoimmune diseases (such as systemic lupus, type 1 diabetes, etc.), inflammatory bowel disease, systemic inflammation and infection as well as metabolic syndrome and NAFLD[35]. To our knowledge, this is the first study to demonstrate that manipulation of the microbiome is associated with an improvement in intestinal permeability in patients with a syndrome which has been associated with intestinal microbiome changes, other than *C. difficile* infection. This raises the possibility of FMT or other microbiome-altering techniques being able to prevent complications of increased intestinal permeability[35]. In the future, screening patients for elevated gut permeability may be a method to select patients likely to benefit from FMT.

This is the first FMT study to monitor changes in hepatic PDFF longitudinally after FMT. No improvement in the hepatic PDFF was detected on MRI testing at 6 months' post FMT. This may be a result of changes in the microbiome associated with allogenic FMT not persisting for as long as 6 months. The reason for choosing this time point was to detect a meaningful change in hepatic PDFF. It is notable however that at 6 weeks post-FMT the

NEFA (Supplementary Figure 5.25) and at 6 months' post-FMT the total cholesterol: HDL cholesterol ratio (Supplementary Figure 5.26) improved in the allogenic group.

There has been very limited data assessing long term changes in the gut microbiome after a single FMT in patients who are transplanted for a condition other than *C. difficile*[36,37]. Arguably, the microbiome alterations are more easily obtained in *C. difficile* patients where microbiota diversity and richness at baseline are extremely low[38]. It may be a key reason for the sustainability of the transplant in that condition[36,37]. On the other hand, repeated FMT for other conditions may require repetitive interventions to prevent reversion of the microbiome to baseline. This may explain why persistent use of a probiotic led to improvement of hepatic PDFF at 6 months[39], but the single FMT in this study failed to do so. It is also notable that although our study did not discern changes in specific taxa within the fecal microbiota with allogenic transplant, we did however observe a trend towards an increase in the fecal microbiota diversity in patients who had improvement in intestinal permeability (Figure 5.5). The lack of changes in specific bacterial taxa may reflect that the FMT was administered into the duodenum, but microbiome analysis was limited to stool specimens. Analysis of the stool may not reflect changes in the microbiome of the small bowel or proximal colon. In fact, a recent FMT study which used duodenal administration in metabolic syndrome noted this phenomenon with changes in the small intestinal bacterial taxa which were not reflected by changes in the specific fecal bacterial taxa and also not associated with a change in fecal microbial diversity[21]. Alternative combination approaches for FMT administration such as pretreatment with antibiotics or co-administration via the colonic and duodenal route may need to be explored. Another hypothesis would be that the changes we observed in the small intestinal permeability of the allogenic group were not dependent on specific bacterial engraftment. Sterile fecal filtrates have been shown to successfully treat patients with *C. difficile* infection[40]. This may be on the basis of bacterial metabolites or possibly bacteriophages. Further studies to clarify these hypotheses are warranted.

Limitations of this study include that by random chance the autologous and allogenic groups differed regarding fibrosis staging, intestinal permeability, and biochemical data. This was likely due to the small sample size of this study. Additionally, as fibrosis staging was done prior to enrollment to the study as part of routine care, three different methods of staging fibrosis were used and more than one pathologist was involved in the evaluation of biopsies; this may have resulted in interobserver variability regarding NASH diagnosis, grading activity and staging fibrosis. However, our outcome measures did not involve comparing pre- and post-treatment histological findings, so this would not affect the study outcome. A single radiologist read all MRI's for hepatic fat content both pre- and post-treatment. This adds to the reliability of our secondary outcome of PDFF. Finally, a single low dose FMT was used, and this may not have been a sufficient dose of bacteria to cause significant changes to the gut microbiome. A bowel prep was given to patients before the FMT, but in the future antibiotics may be used to improve donor engraftment.

In conclusion, duodenoscopy administered FMT did not improve insulin sensitivity (measured via HOMA-IR) or hepatic PDFF in NAFLD patients, but it did contribute to repair of intestinal permeability. The use of FMT warrants further investigation to modulate diseases associated with increased intestinal permeability including NAFLD.

ClinicalTrials.gov NCT02496390

5.5 References

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Chapter 6

6 Safety, intestinal permeability, and changes in fecal microbiota composition following fecal microbiota transplantations in patients with multiple sclerosis

6.1 Introduction

Multiple sclerosis is an autoimmune, inflammatory, demyelinating disease of the central nervous system influenced by genetic susceptibility and some environmental factors. Patients with MS experience loss of motor function and vision, bladder and bowel dysfunction, muscle spasms, and cognitive impairment, which significantly decrease their quality of life. The life expectancy of these patients has been estimated to be decreased by 7 years on average[1].

There are no existing therapies available to stop the progression of MS. Current medications are used to slow the progression of MS, manage relapse symptoms, and speed up recovery from relapse[2]. Investigation into novel therapies is needed. Past studies have shown that MS patients have alterations in their gut microbiota composition compared to healthy individuals. The most common trends that have been observed are a decrease in butyrate-producing bacteria including *Clostridium* cluster XIVa, *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Lachnospiraceae, Ruminococcaceae, Fusobacterium* and *Butyricimonas*[3-5]. Butyrate is preferentially used by intestinal epithelial cells as a source of energy and it promotes intestinal barrier integrity[6]. A decrease in butyrate may cause an increase in intestinal permeability, and this may allow microbial and dietary antigens to pass through the intestinal epithelium and trigger autoimmune responses in the host. Rumah et al. identified an MS patient with epsilon toxin (ETX)-producing *Clostridium perfringens* Type B[7]. It can disrupt the blood-brain barrier and bind myelin making it a potential MS trigger. Ten percent of MS patients in the study had antibodies specific to ETX compared to 1% of the controls[7]. Molecular mimicry could also be a

possible factor contributing to the development of MS as *Pseudomonas* peptides are capable of activating myelin basic protein (MBP)-specific T-cell clones from MS patients[8].

Due to the links between MS and the gut microbiome, fecal microbiota transplantation (FMT) is being considered as a possible treatment for MS. We hypothesized that FMTs from healthy donors without a personal or family history of autoimmune diseases would be safe and well tolerated, alter the balance of cytokines in the peripheral blood to become more anti-inflammatory, and alter the microbiota composition of MS patients.

6.2 Methods

6.2.1 Patient Recruitment and Group Randomization

Ten patients (three males and seven females) with RRMS (n=9) and SPMS (n=1) were recruited to the study between October 2017 to May 2018 at a neurology clinic at University Hospital in London, ON, Canada. [ClinicalTrials.gov (NCT03183869)] The study was approved by Health Canada as well as Western University's Research Ethics Board (REB: 109306). All patients provided written informed consent. Patients were randomly assigned to either the early (n=4) or late intervention group (n=6). Early intervention patients received one FMT per month for six months in the first six months of the study and were monitored for six months post-FMTs. Late intervention patients were recruited to the study and were monitored for six months prior to intervention. They then received one FMT per month for six months in the last six months of the study. Patients were randomly assigned to receive FMTs from either Donor 1 (n=4) or Donor 2 (n=6).

6.2.2 Donor Selection

Potential donors were screened using our previously published protocol[9] with the additional exclusion criteria of any family or personal history of autoimmune diseases. Two donors were selected for this study. Donor 1 provided FMTs to four patients, Donor 2 provided FMTs to six patients. Donor 2 in this study was Donor 1 from the NAFLD-FMT clinical trial.

6.2.3 Fecal Microbiota Transplant

Stool samples of 50-70 g were collected from donors and stored as whole stool at -80 °C for less than three months. The stool samples were thawed in a 37 °C water bath for one hour prior to preparation of the enema. Two hundred and twenty millilitres of saline and 50-70 g of donor stool were placed inside of a BA614/STR filter bag (Seward, Islandia, NY) and were mixed using the Stomacher® 400 Circulator (Seward, Islandia, NY) at 230 rpm for 30 seconds. The filtered material was then transferred into an AMSure® Enema Bag (Amsino, Pomona, CA). The enema was prepared thirty minutes before the scheduled FMT and was stored at room temperature until the procedure took place.

6.2.4 Blood, Urine, Vitals

Routine blood work, urinalysis, and vitals were taken at each visit (once per month for up to twelve months). Blood and urine were collected, and vitals were taken prior to administering the FMT at each appointment. A summary of tests performed can be found in Supplementary Tables 6.1-6.3. These samples were collected and analyzed as a part of standard of care at St. Joseph's Health Care, London, ON.

6.2.5 EDSS and MRI

Clinical and radiological signs of disease activity or progression were measured using the EDSS[10] once per month for twelve months and MRI at baseline, six months, and twelve months. EDSS was administered by Dr. Ana Wing and Dr. Marcelo Kremenchutzky at St. Joseph's Health Care, London, ON. MRI data was reviewed by Dr. Sarah Morrow at University Hospital, London, ON.

6.2.6 Intestinal Permeability

Patients were asked to drink a solution of 5 g of lactulose (Calbiochem®, EMD Millipore Corp., Billerica, MA), 2 g of mannitol powder (BDH®, VWR analytical, Mississauga, ON), 1.5 g of Kool Aid (Kraft Foods, Ingleside, ON), 100 g of sucrose, and 450 mL of tap water the evening before their baseline, six month, and twelve month follow-ups. The

subjects were asked to collect all the urine that they passed throughout the night and morning of their appointment and store it in a urine collection bottle. This bottle was brought to the clinic, the total volume of urine was recorded and then aliquoted into 10 mL. Concentrations of lactulose, mannitol and sucrose were determined using high performance liquid chromatography[11]. Urine samples were sent to the lab of Dr. Jon Meddings in Calgary, AB, at the end of the study to be analyzed.

6.2.7 Fecal sample collection

Fecal samples were collected from patients at each time point using a toilet paper sampling method[12]. Briefly, patients collected a visibly soiled piece of toilet paper after passing a stool 1-3 days before their scheduled appointment. The subjects placed the fecal sample in a Fisherbrand™ Opaque Sterile Sampling Bag (Fischer Scientific, ThermoFisher Scientific, Mississauga, ON). and brought it to their appointments. The samples were then frozen at - 80 °C until DNA extraction took place. Fecal samples were collected once per month for up to twelve months.

6.2.8 DNA Extraction

DNA from the toilet paper samples was extracted using the DNeasy® Powersoil® HTP 96 Kit (Qiagen, Toronto, Ontario, Canada), as per the manufacturer's instructions with the following modification: A centrifuge speed of 3700 *x* g for 10 minutes was used. Extracted DNA was stored at -20 °C until amplification.

6.2.9 DNA Amplification

The BioMek® 3000 Laboratory Automation Workstation for automated PCR reagent set up was used to load 10 µL (2.3 pmol/µL) of 32 primers (16 left and 16 right) with unique barcodes into 96 well plates. Amplifications of the V4 region of the 16S ribosomal RNA gene were carried out with the primers (5′-3′) ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNxxxxxxxxGTGCCAGCMG CCGCGGTAA and (5′-3′) CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNxxxxxxxxGGACTA

CHVGGGTWTCTAAT (xxxxxxxx is a sample specific nucleotide barcode and the preceding sequence is a portion of the Illumina adapter sequence for library construction). The BioMek® robot was then used to transfer 2 µL of template DNA into the primer containing 96 well plates. Then 20 µL of Promega GoTaq® Colourless Master Mix (Promega, Maddison, WI) was added to the DNA template and primers. The final plate was sealed with a foil PCR plate cover. This plate was placed in the Eppendorf Mastercycler® thermal cycler (Eppendorf, Mississauga, Ontario, Canada), where the lid was kept at 105 °C. An initial warm-up temperature of 95 °C was used for 2 min to activate the GoTaq®. Afterwards, the volumes underwent 25 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. After completion, the temperature of the thermal cycler was held at 4 °C, and amplicons were then stored at -20 °C.

6.2.10 DNA sequencing and data analysis

Amplified DNA was sent to the London Regional Genomics Centre at Robarts Research Institute (Western University, London, Ontario, Canada). The samples were quantified (Quant-it, Life Technologies, Burlington, Ontario, Canada) and pooled at equimolar concentrations. The pooled libraries were cleaned using QIAquick (Qiagen, Germantown, Maryland, USA) and then sequenced using the MiSeq Illumina® platform, with 2 × 250 bp paired-end chemistry. The reads were demultiplexed and filtered using dada2 (version 1.8) and custom R scripts written by Greg Gloor (github.com/ggloor/miseq_bin). The demultiplexed reads will be available at NCBI SRA. Any taxa with less than 3 counts in 30% of the samples were removed. Reads with 97% similarity were grouped into OTUs. Taxonomy was assigned using an RDP classifier provided by the dada2 package and trained against version 132 of the SILVA database. Diversity of the fecal microbiota was quantified based on Shannon's index and was calculated using the Vegan package (github.com/vegandevs/vegan). Compositional distance between communities was quantified using the Aitchison distance with a prior count of 0.5 to avoid taking the log or ratio of zero counts[13]. ALDEx2 was used to identify differentially abundant taxa between different time points[14].

6.2.11 Statistical Analysis

Friedman test was used to compare EDSS at baseline to all subsequent time points. Wilcoxon matched-pairs signed rank test was used to compare biochemical test results in MS patients before and after FMT. Wilcoxon matched-pairs signed rank test was used to compare cytokine concentrations at baseline to subsequent time points. Wilcoxon matched-pairs signed rank test was used to compare gut microbiota diversity of MS patients at baseline to subsequent time points. Mann-Whiney test was used to compare the gut microbiota diversity of donors to MS patients at baseline. Mann-Whitney test was used to compared Aitchison distance of patients that had received FMT to patients waiting to receive FMT. An effect size cutoff of > |3| was used for the ALDEx2 analysis.

6.3 Results

6.3.1 FMT was safe and tolerable in MS patients.

The patients had a mean age of 46.1 ± 10.2 years, the average age of diagnosis was 32.1 ± 8.5 years of age, and the average duration of MS was 15.9 ± 7.7 years. Adverse events were documented and are summarized in Table 6.1.

Table 6.1 Summary of adverse events

Adverse Event	Treatment Group (n=10)			Related to Treatment		
	Mild	Moderate	Severe	Mild	Moderate	Severe
Difficulty swallowing	1	0	0	0	0	0
Yeast Infection	1	0	0	0	0	0
Influenza virus	1	0	0	0	0	0
Diarrhea	2	0	0	0	0	0
Common cold	1	0	0	0	0	0
Nausea	2	0	0	0	0	0
Vomiting	1	0	0	0	0	0
Hypertension	0	1	0	0	0	0
Kidney Stone	1	0	0	0	0	0
Cramping	1	0	0	0	0	0
Abdominal discomfort	1	0	0	0	0	0
Ear infection	1	0	0	0	0	0
Extreme fatigue	1	0	0	0	0	0

Routine blood work and urinalysis were performed, and vitals were taken at each appointment. There were no significant differences in these parameters following the administration of six FMTs (Supplementary Tables 6.1-6.3). At baseline the mean EDSS was 3.5 ± 2.0 (n=10). EDSS was measured at every visit and there was no significant change in EDSS following repeated FMTs (Figure 6.1). MRI was performed at baseline and 6 months as a safety measure since patients may have MRI activity even in the absence of clinical relapse. No new MRI activity was developed following FMTs (data not shown).

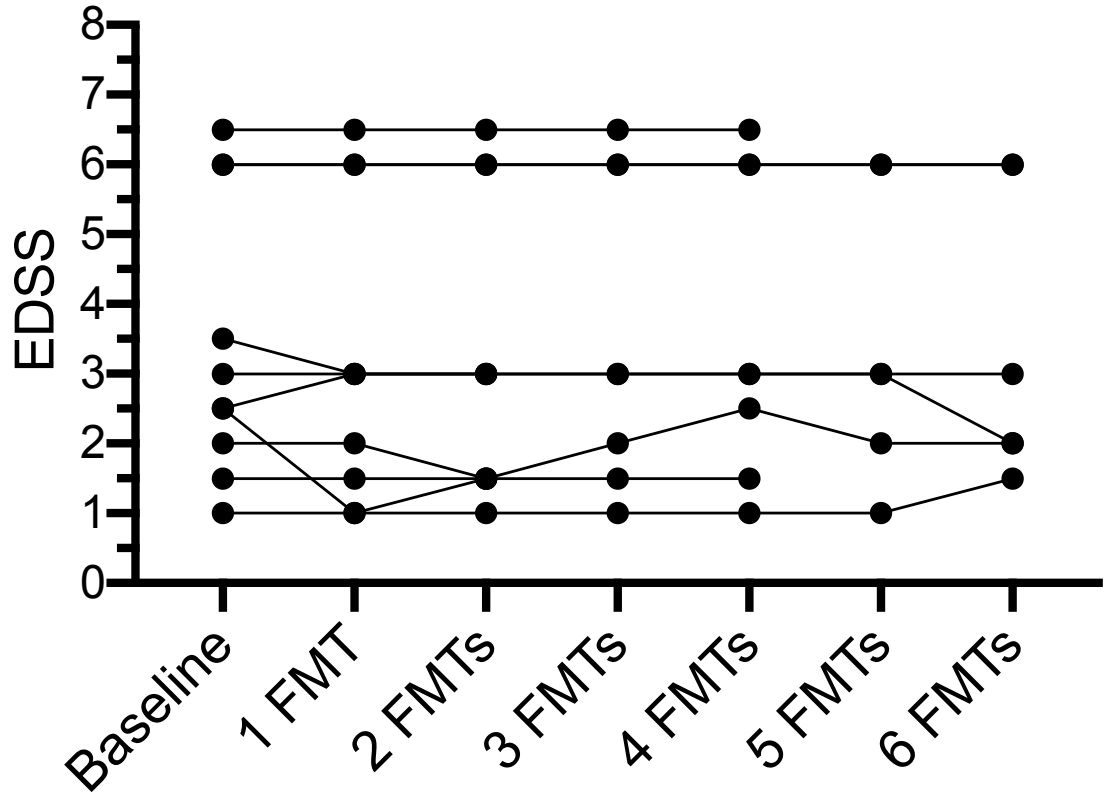

Figure 6.1 EDSS is stable following multiple FMTs. Patients received one FMT per month for six months and EDSS was measured at each visit. Six patients received all six FMTs and ten patients received at least one FMT. EDSS was calculated by the neurologist. Friedman test was used to compare the EDSS at baseline and subsequent time points, p=0.898.

6.3.2 The gut microbiota composition was altered and differed by patient.

Principal component analysis (PCA) plots of the fecal microbiota samples collected from patients that received FMTs from Donors 1 and 2 were constructed. The distance between samples represents how similar in microbial composition two samples are; samples that are close together are more similar in composition than samples that are farther apart. The PCA plots showed that 34.6 % and 28.1 % of the variance was explained in the first two principal components, respectively (Figures 6.2 and 6.3). The largest source of variance was from

individual differences in fecal microbiota composition (Figures 6.2 and 6.3). Donors 1 and 2 did not have a fecal microbiota composition that clustered separately from MS patients (Figures 6.2 and 6.3). Patients that received an FMT had a fecal microbiota composition that still closely resembled their initial profile at baseline compared to other patients or Donors 1 or 2 (Figures 6.2 and 6.3).

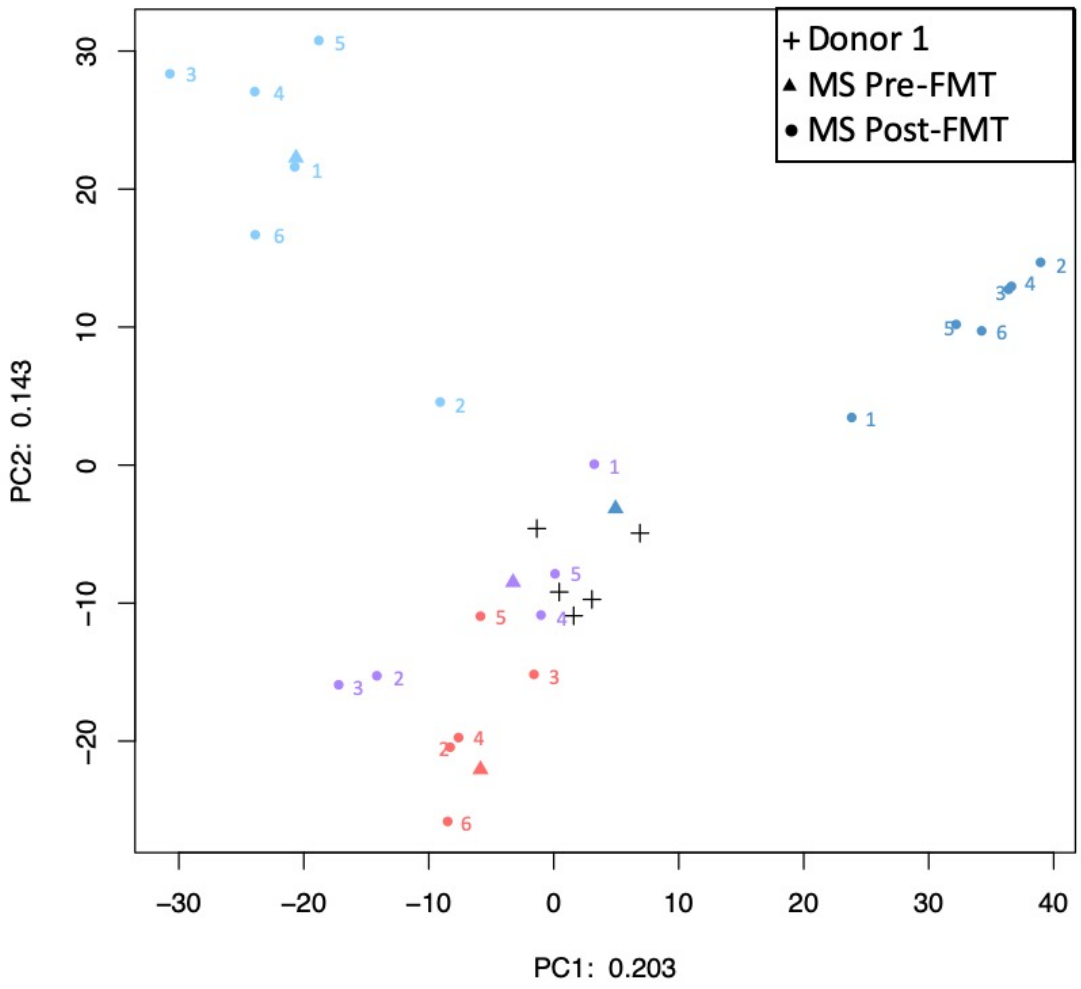

Figure 6.2 Principal component analysis of the fecal microbiota composition following FMT from Donor 1. Fecal samples were collected from patients one to three days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate a PCA in R (version 3.5.3). The first two components explain 34.6 % of the variance. Donor 1 is shown in black (n=1; five time points) and each MS patient is shown in a different colour (n=4). Triangles represent fecal microbiota samples

at baseline. Circles represent fecal microbiota samples collected following FMT. The number indicates how many FMTs had been received at that time point.

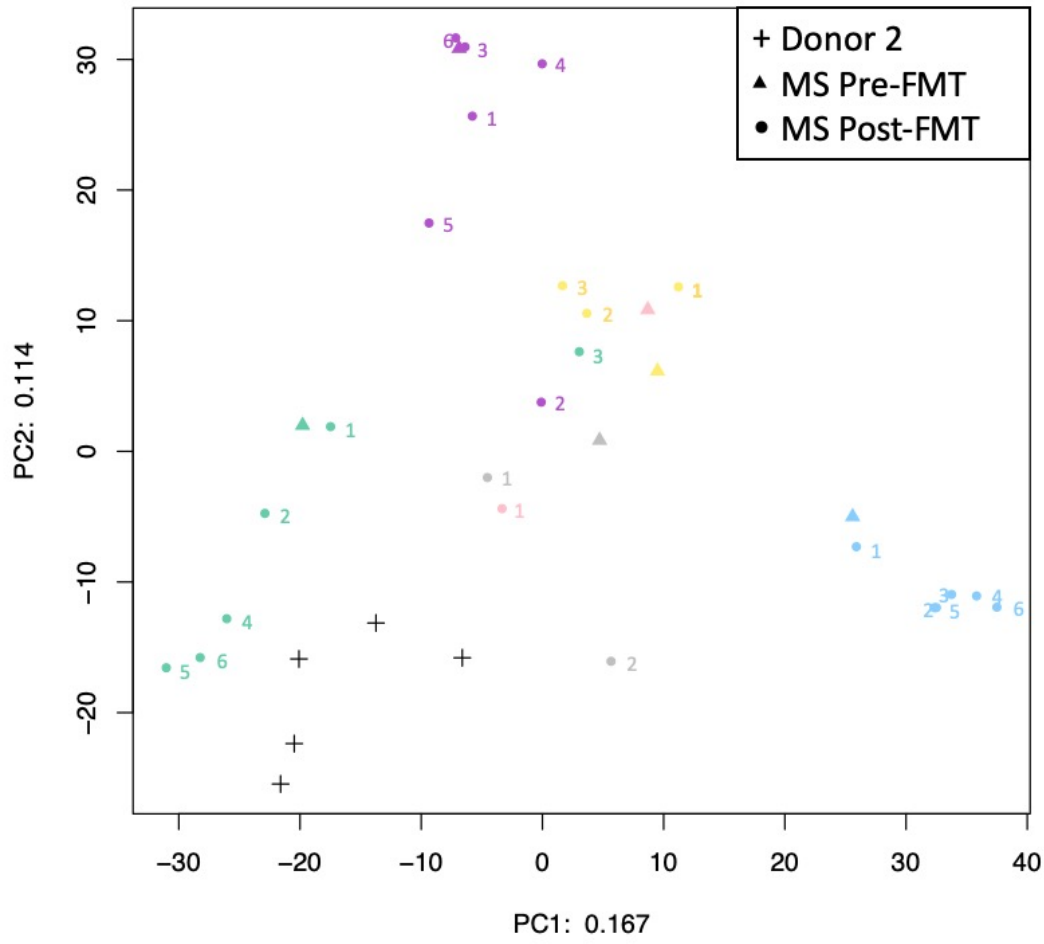

Figure 6.3 Principal component analysis of the fecal microbiota composition following FMT from Donor 2. Fecal samples were collected from patients one to three days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate a PCA in R (version 3.5.3). The first two components explain 28.1 % of the variance. Donor 2 is shown in black (n=1; five time points) and each MS patient is shown in a different colour (n=6). Triangles represent fecal microbiota samples

at baseline. Circles represent fecal microbiota samples collected following FMT. The number indicates how many FMTs had been received at that time point.

Differences between baseline microbiota composition and subsequent time-points were quantified using the Aitchison distance which provides a compositionally robust alternative to standard β-diversity metrics[13]. Aitchison distance increases as the bacterial composition of two samples are less similar to one another. There was no significant increase in the Aitchison distance of allogenic FMT recipient patients after six FMTs compared to MS patients that waited six months before receiving FMTs (Figure 6.4). Shannon's diversity index was also calculated, and the donors were found to have a fecal microbiota diversity that was comparable to MS patients at baseline. MS patients did not undergo an increase in fecal microbiota diversity following repeated FMTs (Figure 6.5).

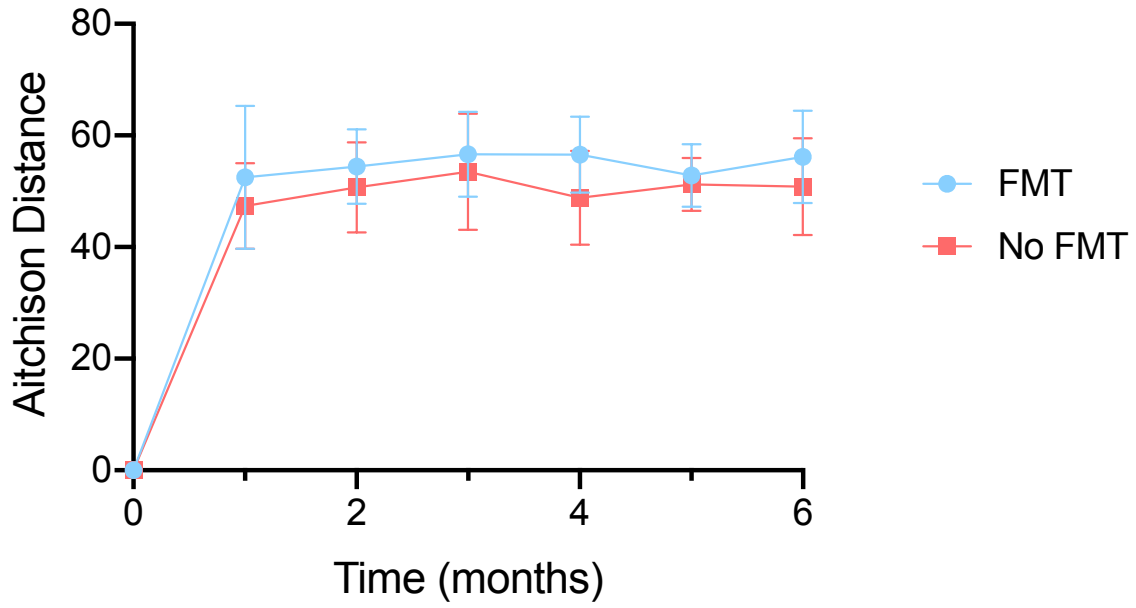

Figure 6.4 The microbiota composition of patients that received an FMT did not change more than patients waiting to receive an FMT. Fecal samples were collected from patients one to three days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. Compositional distance between communities was

quantified using the Aitchison distance with a prior count of 0.5 to avoid taking the log or ratio of zero counts. Data displayed is Aitchison distance from the baseline fecal microbiota composition compared to subsequent time points (1 month, 2 months, 3 months, 4 months, 5 months, and 6 months) pre- (n=8) or post-FMT (n=10). Mann-Whitney t-test was used to compare Aitchison distance of patients who had received an FMT to patients that were waiting for an FMT at each time point.

1 m FMT vs no FMT, p=0.1562 (Mann-Whitney)

2 m FMT vs no FMT, p=0.4079 (Mann-Whitney)

3 m FMT vs no FMT, p=0.3969 (Mann-Whitney)

4 m FMT vs no FMT, p=0.2086 (Mann-Whitney)

5 m FMT vs no FMT, p=0.8357 (Mann-Whitney)

6 m FMT vs no FMT, p=0.3095 (Mann-Whitney)

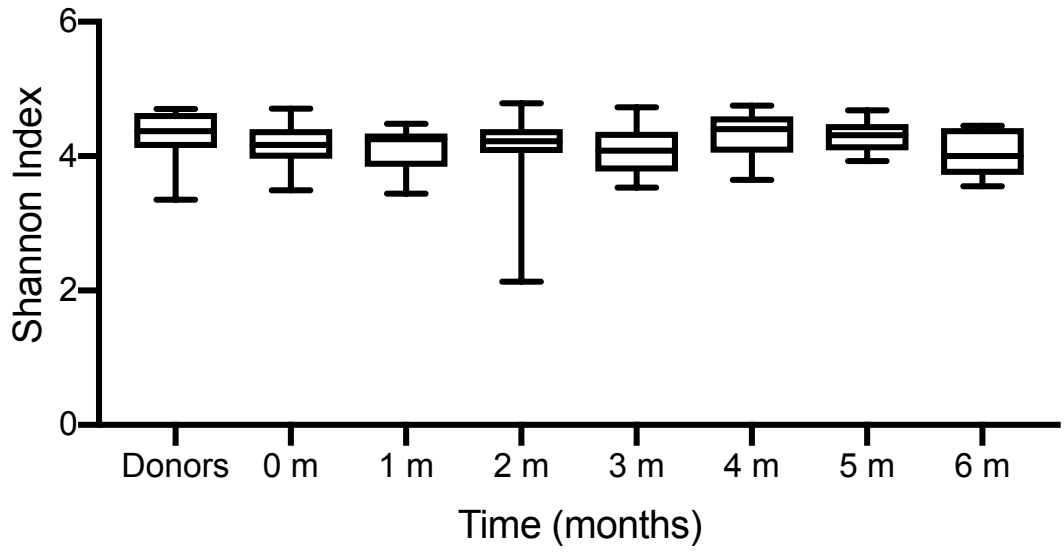

Figure 6.5 Fecal bacterial diversity of MS patients is not different from healthy donors and does not increase following repeated FMTs. Fecal samples were collected from patients one to three days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to calculate Shannon Diversity Index using the vegan package in R (version 3.5.3)

(github.com/vegandevs/vegan). Shannon Diversity Index of MS (n=10) patients at baseline and after 1 month (1 FMT), 2 months (2 FMTs), 3 months (3 FMTs), 4 months (4 FMTs), 5 months (5 FMTs), 6 months (6 FMTs) and healthy donors (n=2) are displayed. Mann-Whitney t-test was used to compare Shannon Diversity Index of healthy donors to MS patients at baseline. Wilcoxon match-pairs signed rank test was used to compare Shannon Diversity Index of MS patients at baseline to subsequent time points.

Donors vs 0 m, p=0.353 (Mann-Whitney)

MS 0 m vs 1 m (n=9), p=0.910 (Wilcoxon match-pairs signed rank test)

MS 0 m vs 2 m (n=9), p>0.999 (Wilcoxon match-pairs signed rank test)

MS 0 m vs 3 m (n=9), p=0.359 (Wilcoxon match-pairs signed rank test)

MS 0 m vs 4 m (n=7), p=0.219 (Wilcoxon match-pairs signed rank test)

MS 0 m vs 5 m (n=7), p=0.156 (Wilcoxon match-pairs signed rank test)

MS 0 m vs 6 m (n=6), p=0.563 (Wilcoxon match-pairs signed rank test)

ALDEx2 was used to identify any differentially abundant taxa between the gut microbiota composition at baseline and after one (n=10), two (n=9), three (n=8), four (n=7), five (n=7), or six (n=6) FMTs (Supplementary Figures 6.1-6.6). There were no significant changes in the composition of the gut microbiota of MS patients following FMTs that were conserved across all study participants (effect size > |3|)

There were differences in the fecal microbiota composition observed at an individual level. A visual inspection was conducted on the bar plots of individual patients to determine what changes in relative abundance occurred throughout the course of the study. These changes are summarized in Table 6.2. Bar plots that show the relative abundance of bacterial genera of each patient before and after FMT are shown in Figure 6.6. A detailed analysis of each individual can be in Supplementary Figures 6.7-6.16. Seven of the ten participants, MK-FMT-001, MK-FMT-004, MK-FMT-007, MK-FMT-008, MK-FMT-010, MK-FMT-011, MK-FMT-012, experienced a noticeable change in their overall microbiota composition following FMTs (Supplementary Figures 6.7, 6.10, 6.11, 6.12, 6.14, 6.15, 6.15) and three did not, MK-FMT-002, MK-FMT-003, MK-FMT-009 (Supplementary Figures 6.8, 6.9,

6.13). Three of the seven patients, MK-FMT-001, MK-FMT-010, MK-FMT-011, had persistent changes in their microbiota that were still present at the end study observation period (Supplementary Figures 6.7, 6.14, 6.15). One patient required three FMTs before a consistent change was observed in their microbiota and the other two patients required four FMTs. The other four patients, MK-FMT-004, MK-FMT-007, MK-FMT-008, MK-FMT-012 had variable microbiota compositions that were different from baseline, but continually changed following subsequent FMTs (Supplementary Figures 6.10 and 6.11) or the duration of follow-up was too short to determine if the changes were persistent (Supplementary Figures 6.12 and 6.16). All patients experienced some change in the relative abundance of *Bacteroides* following FMT. Seven out of the ten participants had consistently decreased relative abundance of *Bacteroides* and the other three patients had an increase in the relative abundance of *Bacteroides* (Table 6.2). Donor 2 was characterized by a large relative abundance of *Prevotella* and three out of five MS patients that received FMTs from Donor 2 had an increase in the relative abundance of *Prevotella* (Figure 6.6). In one patient in particular, MK-FMT-011, it appeared that the increase in relative abundance of *Prevotella* was cumulative with subsequent FMT (Supplementary Figure 6.15). Two patients experienced changes in the relative abundance of *Faecalibacterium*, one increased, MK-FMT-008, and one decreased, MK-FMT-004 following FMT (Supplementary Figures 6.12 and 6.10, respectively).

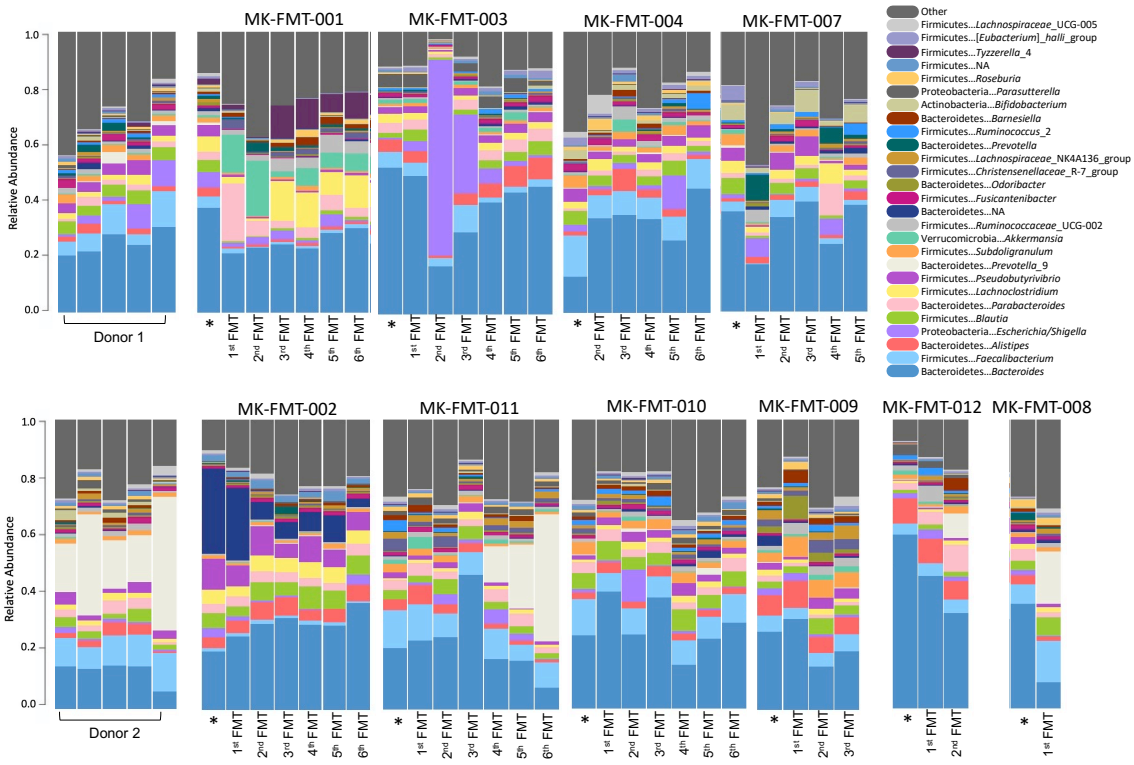

Figure 6.6 Fecal microbiota composition of MS patients following FMTs. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. Four patients received FMTs from Donor 1 and six patients received FMTs from Donor 2. Patients that received FMTs from Donor 1 are in the top row of bar plots. Patients that received FMTs from Donor 2 are on the bottom row of bar plots. * represents the sample taken at baseline before any FMTs were administered. Subsequent time points include 1st FMT, 2nd FMT, 3rd FMT, 4th FMT, 5th FMT, and 6th FMT. One patient that received FMTs from Donor 1 did not submit a stool sample corresponding to the 1st FMT.

Table 6.2 Individual changes in gut microbiota composition following FMT.

Patient	Donor	1st FMT	2nd FMT	3rd FMT
MK-FMT-001	Donor 1	↓ *Bacteroides* ↑ *Akkermansia* ↑ *Parabacteroides* ↑ *Peptoniphilus*	↓ *Bacteroides* ↑ *Akkermansia* ↑ *Parabacteroides* ↑ *Ruminococcus*	↓ *Bacteroides* ↑ *Akkermansia* ↑ *Lachnoclostridium* ↑ *Tyzzerella*
MK-FMT-002	Donor 2	↑ *Bacteroides* ↓ *Escherichia/ Shigella*	↑ *Anaerofilum* ↑ *Bacteroides* ↓ *Escherichia/ Shigella* ↓ Unknown Bacteroidetes	↑ *Bacteroides* ↓ *Escherichia/Shigella* ↓ Unknown Bacteroidetes
MK-FMT-003	Donor 1		↓ *Bacteroides* ↑ *Escherichia/Shigella*	↓ *Bacteroides* ↑ *Escherichia/ Shigella*
MK-FMT-004	Donor 1	↓ *Faecalibacterium* ↑ *Bacteroides*	↓ *Erispelotrichaceae* ↓ *Faecalibacterium* ↑ *Alistipes* ↑ *Bacteroides*	↓ *Erispelotrichaceae* ↓ *Faecalibacterium* ↑ *Bacteroides*
MK-FMT-007	Donor 1	↓ *Bacteroides* ↓ *Bifidobacterium* ↑ *Escherichia/Shigella* ↑ *Porphyromonas* ↑ *Prevotella*		
MK-FMT-008	Donor 2	↓ *Bacteroides* ↓ *Escherichia/Shigella* ↑ *Blautia* ↑ *Faecalibacterium* ↑ *Prevotella*		
MK-FMT-009	Donor 2	↑ *Odoribacter*	↓ *Bacteroides*	↓ *Bacteroides*
MK-FMT-010	Donor 2		↑ *Bacteroides* ↑ *Escherichia/Shigella*	
MK-FMT-011	Donor 2		↑ *Akkermansia*	↑ *Bacteroides* ↑ *Escherichia/Shigella*
MK-FMT-012	Donor 2	↓ *Bacteroides* ↑ *Parabacteroides*	↓ *Bacteroides* ↑ *Lachnoclostridium* ↑ *Parabacteroides* ↑ *Prevotella*	

Patient	4th FMT	5th FMT	6th FMT
MK-FMT-001	↓ *Bacteroides* ↑ *Akkermansia* ↑ *Lachnoclostridium* ↑ *Tyzzerella*	↓ *Bacteroides* ↑ *Akkermansia* ↑ *Lachnoclostridium* ↑ *Parabacteroides* ↑ *Tyzzerella*	↓ *Bacteroides* ↑ *Akkermansia* ↑ *Lachnoclostridium* ↑ *Tyzzerella*
MK-FMT-002	↑ *Bacteroides* ↓ *Escherichia/Shigella* ↓ Unknown Bacteroidetes	↑ *Anaerofilum* ↑ *Bacteroides* ↓ *Escherichia/ Shigella* ↓ Unknown Bacteroidetes	↑ *Bacteroides* ↓ *Escherichia/Shigella* ↓ Unknown Bacteroidetes
MK-FMT-003	↓ *Bacteroides*	↓ *Bacteroides*	↓ *Bacteroides*
MK-FMT-004	↓ *Erispelotrichaceae* ↓ *Faecalibacterium* ↑ *Bacteroides*	↓ *Erispelotrichaceae* ↓ *Faecalibacterium* ↑ *Bacteroides* ↑ *Escherichia/Shigella*	↓ *Erispelotrichaceae* ↑ *Bacteroides*
MK-FMT-007	↓ *Bacteroides* ↓ *Bifidobacterium* ↑ *Lachnoclostridium* ↑ *Parabacteroides* ↑ *Porphyromonas* ↑ *Prevotella*		
MK-FMT-008			
MK-FMT-009			
MK-FMT-010	↑ *Bacteroides*	↓ *Bacteroides*	
MK-FMT-011	↓ *Bacteroides* ↑ *Prevotella*	↓ *Bacteroides* ↑ *Escherichia/ Shigella* ↑ *Prevotella*	↓ *Bacteroides* ↑ *Prevotella*
MK-FMT-012			

6.3.3 Abnormal intestinal permeability was improved following FMT in MS patients

Five patients had their small intestinal permeability assessed at both baseline and following all six FMTs. Two patients had abnormal small intestinal permeability at baseline (>0.025 Lactulose:Mannitol) (Figure 6.7). Both patients had normal small intestinal permeability following six FMTs (Figure 6.7).

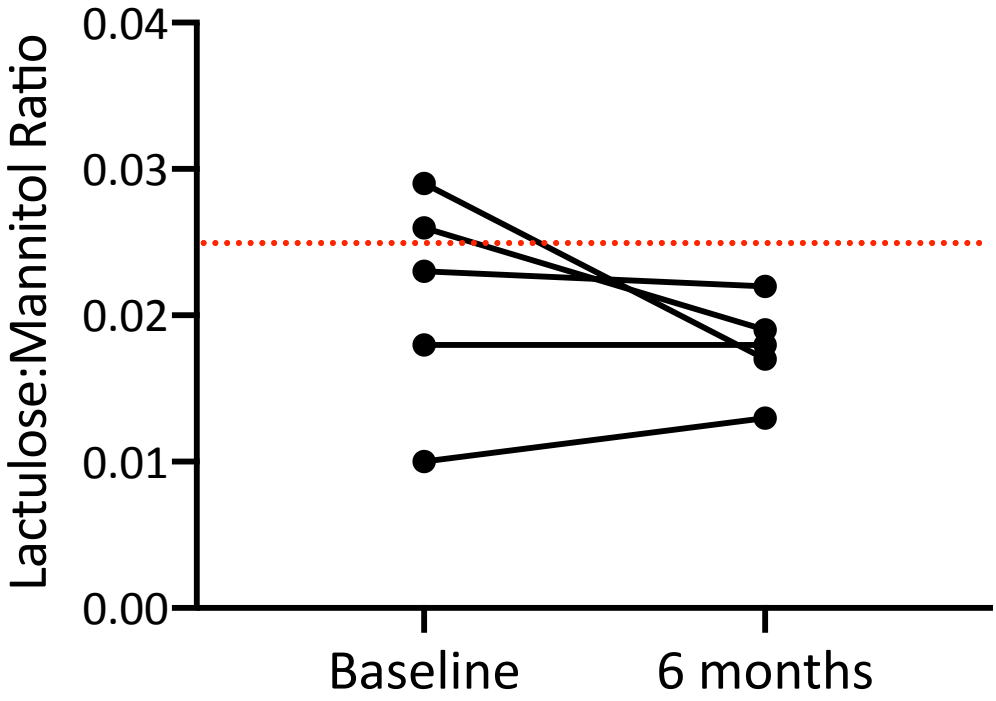

Figure 6.7 Small intestinal permeability remained stable following repeated FMTs. Patients were asked to drink a solution of 5 g of lactulose, 2 g of mannitol powder, 1.5 g of Kool Aid, 100 g of sucrose, and 450 mL of tap water the evening before their appointments at baseline and following six FMTs. The subjects were asked to collect all the urine that they passed throughout the night and morning of their appointment and store it in a urine collection bottle. Concentrations of lactulose, mannitol and sucrose were determined using high performance liquid chromatography. Five patients had data for both baseline and following six FMTs (n=5). Wilcoxon matched-pairs signed rank test was used to compare lactulose:mannitol at baseline and six months (p=0.375). Abnormal intestinal permeability is > 0.025 lactulose:mannitol, which is represented by the red dotted line.

6.4 Discussion

This study was the largest to date of FMT therapy for MS patients. Overall, this study showed that FMT was safe in MS patients and that some patients experienced a change in the composition of the gut microbiota. The most common being the relative abundance of *Bacteroides*, and these changes required multiple FMTs to persist. *Prevotella* was found to

increase in relative abundance in three of five patients that received an FMT from Donor 2. Adverse events were documented throughout the course of the study and none were found to be related to the FMT therapy given (Table 6.1). Blood chemistry, hematology markers, and vitals, were taken at each appointment and there were no significant changes found in any of the parameters that were measured (Supplementary Tables 6.1-6.3). There were no significant changes observed in patients' MRIs or EDSS over the duration of the study (Figure 6.1). Normal small intestinal permeability was found to be restored in both patients that had elevated small intestinal permeability (Figure 6.7). This data supported that FMT was a safe and well-tolerated intervention for MS patients.

Aitchison distance was measured and the composition of the gut bacteria at baseline, before any FMTs, was compared to subsequent time points. A higher value of Aitchison distance would indicate that the composition of gut bacteria at that time point was more different in composition compared to the baseline sample it was being compared to. There was a trend that patients waiting for an FMT had a lower increase in Aitchison distance over time than those who received an FMT, but this was not statistically significant, likely due to the small sample size (Figure 6.4).

An increase in fecal diversity of MS patients following FMT was not observed (Figure 6.5). The MS patients had fecal diversity similar to that of the healthy donors so it would be unlikely that there would be an increase in fecal diversity if these patients did not have low fecal diversity at baseline. Additionally, a review of the literature did not find that low fecal diversity has been found as an alteration in the microbiome of MS patients[15].

Overall, there were no conserved changes in the fecal microbiota composition of MS patients following an FMT from a healthy donor (Table 6.2). However, at an individual level, there were patients that experienced a change in the gut microbiota composition following FMT. Seven patients had a change in their microbiota composition (Supplementary Figures 6.7, 6.10, 6.11, 6.12, 6.14, 6.15, 6.15). Three of these patients had persistent changes in the microbiota composition after three (Supplementary Figure 6.7) or four FMTs (Supplementary Figures 6.14 and 6.15), which led us to believe that multiple

FMTs were necessary to drive long-lasting changes in the microbiome. Three patients did not appear to have an alteration in the composition of their fecal microbiota following FMT, indicating that the donor microbiota may not have engrafted in these patients (Supplementary Figures 6.8, 6.9, 6.13). Two patients had variable microbiota compositions over time, and it was not apparent what changes were due to FMT or natural fluctuation in their microbiota composition (Supplementary Figures 6.10 and 6.11).

Three patients that received FMTs from Donor 2 had an increase in the relative abundance of *Prevotella* and this persisted for the duration of observation (Supplementary Figures 6.12, 6.15, and 6.16). *Prevotella* was the genus in highest relative abundance in Donor 2 and it has been thought to be associated with health because it is found in higher relative abundance in people who eat a vegetarian diet[16]. *Prevotella* has been found to be in higher abundance in healthy controls versus MS patients[17,18], but alternatively it has been found that a higher relative abundance of *Prevotella* can be related to an increase risk of developing rheumatoid arthritis[19], another autoimmune disease. The relative abundance *Bacteroides* was altered in all patients after receiving an FMT. Seven out of ten patients had a decrease in the relative abundance *Bacteroides* following FMT. This change could be because the hosts' *Bacteroides* was displaced by the FMT or it is possible that this decrease was due to the addition of the donor microbiota making the hosts' *Bacteroides* appear less abundant because this is compositional data. *Bacteroides* has not been shown to be associated with MS.

One patient had increased *Akkermansia* following FMT (Supplementary Figure 6.7) and *Akkermansia* has been shown to be elevated in MS patients compared to healthy controls, where it may be promoting the activation of pro-inflammatory T-cells[17]. This patient also had an increase in *Parabacteroides* following FMT and this genus has been found to be increased in healthy controls compared to MS patients[5,18]. Another patient had an increase in the relative abundance of *Escherichia*/*Shigella* following two FMTs and decreased after the fourth FMT (Supplementary Figure 6.9); this patient had diarrhea in the days leading up to their second FMT and may not have been related to MS. This patient also had a

history of recurrent yeast infections and reported subsequent yeast infections following the FMTs. Two patients experienced changes in the relative abundance of *Faecalibacterium*, one increased and one decreased following FMT (Supplementary Figures 6.12 and 6.10, respectively). *Faecalibacterium* has been found to be decreased in MS patients[3,4] and since it is a known butyrate producer, FMT may be a beneficial method to increase the prevalence of *Faecalibacterium* and increase the concentration of butyrate in the gut. An increase in the relative abundance of butyrate producers, such *Faecalibacterium* species, through FMT may be the driving cause of improved gut barrier function[20].

Two of five MS patients had increased intestinal permeability (Figure 6.6). There have been several studies that have found a decrease in butyrate producing bacteria in MS patients compared to healthy controls[3-5] and therefore it has been hypothesized that patients with MS would have increased small intestinal permeability. A review of the literature shows that 20%-73% of MS patients have abnormal small intestinal permeability, using the same methodology of measuring permeability as this study[21,22]. This was comparable to the 40% (2/5) of patients in this study that had abnormal small intestinal permeability. Both patients with abnormal intestinal permeability improved to within the normal range (<0.025 lactulose:mannitol) following FMT (Figure 6.6). A study with a larger sample size is needed to determine what changes in the microbiota may have caused this effect.

Strengths of this study include that the microbiota of MS patients was followed for six months without any microbiome intervention as well as for six months following FMT. To our knowledge, this is the first longitudinal study of the microbiome of MS patients and shows that the gut microbiota composition of MS patient can fluctuate over time without microbial intervention. Also, repeated FMTs were administered in this study to ensure that changes in the gut microbiome persisted over time. Maintenance FMTs have proved to be beneficial at improving the efficacy of FMT for treating rCDI[23,24] and were used in a previous FMT study involving three MS patients[25]. One of the limitations of this study was that patients did not receive a bowel prep or antibiotics prior to the initial FMT procedure, and this potentially limited the ability of the donor microbiota to colonize the MS patients.

Additionally, the differences in fecal microbiota composition between individuals were larger than the changes in composition caused by administration of the FMT from a healthy donor, which made it difficult to determine what changes in the composition of bacteria were a result of an FMT.

Overall, FMT was well tolerated and has the ability to alter the gut microbiota composition of MS patients, the changes in composition are variable between patients, and these alterations can persist with repeated FMT. Larger studies will be required to assess the efficacy of this intervention in the treatment of MS.

6.5 References

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Chapter 7

7 General Discussion

7.1 Experimental Approach

While significant research has gone into NAFLD and MS, both diseases continue to increase in prevalence each year. Exercise and diet are proven methods of treating and preventing NAFLD, while the cause of MS is poorly understood, and curative measures have not been found. The increase in prevalence of these conditions, as well as the reduction in quality of life that can be faced by these patients as these diseases progress, has led to the need of novel therapies. As both conditions have been shown to have alterations in the gut microbiome, FMT has been proposed as a potential treatment.

While FMT has become more widely used, questions must be raised about the safety of donor selection and stool processing. The first goal of this thesis was to identify optimal methods to select healthy donors, and store and prepare fecal matter for FMT that result in the highest yield of viable bacteria, as this may be related to successful outcomes. Stringent donor screening criteria were developed. The resultant FMT material was then tested in FMT clinical trials with patients with NAFLD or MS. This work provided valuable information about the role of the gut microbiome in these diseases, potentially through modulating intestinal permeability, which improved in patients that had abnormal intestinal permeability at baseline. This work justifies new larger studies to provide further information about how intestinal permeability is altered with FMT and if these benefits can be applied to patients with other conditions that are associated with abnormal intestinal permeability.

7.2 Main Findings

Diluents from published studies[1-6], including water, saline, and 10 % glycerol, as well as stored as whole stool, were used to store samples at 4 °C for up to 7 days to test if samples could be stored fresh longer than the then current clinic practice of 4 hours. Samples were

also stored at -20 ° C and -80 °C long-term for 3 months. All samples experienced the greatest change in the composition of viable bacteria during the initial collection of the stool sample (Chapter 2; Figure 2.4a), which was approximately an hour or less in ambient air. Increases in the relative abundance of *Alistipes, Bacteroides,* and *Parasutterella* occurred during sample collection, meaning that these bacteria survived better than others, and therefore increased in relative abundance. Another study of FMT material stability noted that *Alistipes* increased in relative abundance after oxygen exposure and *Faecalibacterium, Subdoligranulum, Eubacterium hallii, Eubacterium rectale, Roseburia,* and *Anaerostipes* decreased[7]. These findings are interesting and add to the literature. It is difficult to determine how these changes in composition of the viable fecal microbiota would affect outcomes of FMT, as the composition of bacteria necessary for successful FMT has yet to determined. At a future date, when more is known about the essential bacteria required for FMT, results of this work can be used to determine if those bacteria are affected by storage and preparation. If a study showed lack of efficacy of FMT, then the composition of stool used could be compared to the results of this work to see if storage and handling impacted efficacy.

Suspending samples in water resulted in an immediate and significant loss of total anaerobes, and the same effect was not observed when samples were suspended in saline or 10% glycerol (Figure 2.2). It was suspected that the initial drop in viability in samples stored in water was due to the hypotonic nature of water that caused cell lysis. Storage in saline and water resulted in greater losses of viable bacteria when samples were stored at -20 °C and -80 °C (Figure 2.3 and 2.4, respectively). Saline and water did not offer any cryopreservative benefits and likely form more ice crystals during freezing than suspension with 10 % glycerol, or whole stool, which has a lower liquid content. Storage at -80°C suspended in 10 % glycerol or as whole stool resulted in the highest viability (Figure 2.4) and diversity of viable bacteria remained stable in these samples (Figure 2.7).

With the emergence of FMT capsules that occurred during this project, the stability of FMT capsules was also investigated. FMT capsules were prepared using a modified

protocol from Kao et al.[6]. Their protocol had two separate freeze-thaws of the fecal material to form the capsules, and the protocol for this project fully formed the capsules before freezing to avoid a second freeze-thaw that may have impacted bacterial viability. The composition and concentration of viable bacteria was tested on fresh stool, immediately after encapsulation, and throughout storage at -80°C to examine the effects of capsule preparation, freeze-thaw, and storage at -80 °C. The largest decrease in bacterial viability occurred during the encapsulation process (Figure 3.2). Following this, viability was stable throughout storage (Figure 3.2). There were some differentially abundant bacteria that were identified to increase or decrease in relative abundance throughout storage and these varied by donor (Table 3.1). This is consistent with other studies that the stability of stool varies by donor[7,8]. The composition of the fecal microbiota of individual donors remained distinct throughout storage (Figure 3.3), despite the small changes in composition that were observed. This was important, as a significant change in the composition of bacteria being delivered during FMT may increase the risk of developing phenotypes of diseases with known alterations to the gut microbiome. After the appropriate method of storing and preparing fecal material for FMT was determined, the selection of FMT donors took place for the FMT clinical trials.

Forty-six PDs offered to be tested, all of whom believed that they met the inclusion criteria. They underwent stringent screening for infectious and gastrointestinal diseases, and personal and family history of a variety of diseases with implications to the gut microbiome. Twenty-three of the 46 potential donors met exclusion criteria during pre-screening. The most common reason for exclusion being a BMI > 25. Eighteen of the remaining 23 PDs failed the blood, urine, and stool screening for infectious diseases and biochemical tests. The most common reasons for exclusion at this step were the detection of *B. hominis, D. fragilis,* and *H. pylori.* Detection of *B. hominis* and *D. fragilis* were found by others to be leading reasons for exclusion[9]. Overall, 11% (5/46) PDs qualified to donate, which was at the lower end of reported rates of 10%-37% [9-12]. Four of the five donors were excluded after two traveled to tropical countries and two experienced acute gastroenteritis after screening. All four donors declined to be rescreened after these events. This is in

agreement with other studies have mentioned the difficulty in retaining donors after initial successful screening[10,12]. The overall low acceptance and retention rates of PDs led to an increased cost of finding a suitable donor, as it cost approximately $15 190 USD to find a single donor. This led to our recommendation that a centralized stool bank be established as individual FMT clinics would likely be unable to fund their own donor screening programs, and to ensure that stool was available when patients came in for transplantation.

After FMT donors were identified that met the inclusion criteria for extended screening for metabolic related diseases, the NAFLD-FMT clinical trial commenced. Twenty-one patients with NAFLD were randomized to receive an FMT from a thin and healthy donor (n=15; allogenic FMT) or their own stool (n=6; autologous FMT). The primary outcome of this study was insulin resistance, as measured by HOMA-IR. Previous work documented that insulin sensitivity could be improved in patients with metabolic syndrome when given an FMT from a thin and healthy donor[13,14], but this study was not able to reproduce these findings (Figure 5.1). This could have been due to using different FMT donors, or measuring insulin resistance using HOMA-IR, instead of the more sensitive insulin clamp technique used by Vrieze et al.[13] and Kootte et al.[14], which was not available in London, ON or the surrounding area. Secondary outcomes of this study were magnetic resonance imaging-derived proton density fat fraction (PDFF) and small intestinal permeability. PDFF was not significantly different than baseline 6 months post-FMT (Figure 5.2). Seven of the fifteen patients in the allogenic FMT group had abnormal intestinal permeability (>0.025 lactulose:mannitol) before FMT and all seven patients experienced a reduction in small intestinal permeability following treatment (Figure 5.3a).

There were no differentially abundant bacteria identified when baseline fecal microbiota compositions of all allogenic or autologous FMT recipients were compared to subsequent time points. Differences in the fecal microbiota composition of FMT recipients varied by individual (Figures 5.6 and 5.7), and therefore statistical analysis could not identify any differentially abundant bacteria that were consistent amongst all group members. This was a surprising finding as FMT is thought to result in significant changes to the gut microbiota

composition. Metagenomic analysis may be required in the future to be able to detect these changes. There was a trend that fecal microbiota diversity increased in patients that had improved small intestinal permeability, but it was not statistically significant (Figure 5.5). Contrary to the literature[14], HOMA-IR non-responders had a significant increase in fecal microbiota diversity following FMT (Figure 5.4).

Based on the improvement in small intestinal permeability that was observed in the NAFLD patients, a subsequent clinical trial was designed and implemented for another condition, MS, with suspected gut microbiome differences and abnormal gut barrier function. Ten patients with MS were recruited to this study and randomized to either an early or late intervention group. Patients in the early intervention group received one FMT per month for the first six months of the study and were observed once per month for the final six months of the study. Patients in the late intervention group served as a control group and were observed once per month for the first six months of the study and then received one FMT per month for the last six months of the study. Due to the premature death of the principal investigator, this study was stopped, and only six of the ten patients received all six FMTs. As there have been no prior publications of FMT in MS patients in peer-reviewed journals, the primary outcomes of this study were safety, including: EDSS, MRI activity, and blood, urine, and stool biochemical tests.

There were no significant changes in EDSS following FMT (Figure 6.1) nor new MRI activity developed following FMT (data not shown). Based on these findings, it was concluded that FMT was well tolerated and safe in MS patients. The fecal microbiota composition of patients was followed before and after FMTs, and similar to the NAFLD study, there were no differentially abundant bacteria identified using statistical measurements between baseline fecal microbiota compositions and subsequent timepoints. Each individual experienced different changes to the composition of their fecal microbiota. Donor 2 in this study was characterized by their high relative abundance of *Prevotella,* and three out of five MS patients who received this donor's stool had an increase in the relative abundance of *Prevotella* that appeared to be cumulative with subsequent FMTs. Therefore,

it was concluded that FMT could to alter the fecal microbiota of MS patients. Five patients completed the lactulose:mannitol urine test to assess small intestinal permeability before and after all six FMTs. Two patients had abnormal small intestinal permeability (>0.025 lactulose:mannitol) and both improved following the full course of FMTs.

7.3 Implications of Findings

It has yet to be determined what is the full importance of bacterial cell viability in FMT treatments, but it is likely that the establishment of the donor microbiota in the recipients would be more challenging with reduced bacterial cell numbers. Both studies of the stability of FMT material identified the need to collect and process fecal samples in an anaerobic environment to try and prevent the initial loss and change in composition of viable bacteria that occurs during sample collection and processing before storage. The use of anaerobic jars to collect samples has recently been implemented by one research group to try and mitigate the effects of oxygen exposure during sample collection[8]. While there are practical limitations of using an anaerobic chamber to manufacture the material, as discussed in Chapter 3, the preservation of viable bacteria is critical as the necessary components of a successful FMT have yet to be identified.

Direct application of this research is already occurring in the London, ON, FMT clinic. At the start of this project, only fresh fecal material, collected within four hours, was being used for FMT. Now whole stool can be refrigerated for longer than 4 hours at 4 °C. This was the method used for the NAFLD-FMT study, as the study began before the long-term storage results had been collected. It was then shown that the viability of whole stool was stable for up to three months at -80°C and the clinic began using these methods of collecting stool, which were implemented in the MS-FMT study. Without this research, it would be incredibly difficult to schedule donors to drop off fresh samples within the four-hour window before a patient's appointment and prepare the FMT. These methods allow the clinic to bank FMT material to use at a later date that still contained a similar number and composition of viable bacteria compared to fresh stool.

When this project began, the use of FMT capsules for rCDI had just recently been published[15]. Until then, FMTs were predominantly administered using nasojejunal[16], enema[1,16], or colonoscopy[3,17-19]. As the project progressed, research became available that FMT capsules were efficient at resolving rCDI[4,6,15,20], that they were more appealing to patients[6], and more cost efficient[6]. Thus, the stability of FMT capsules was also investigated and the concentration of viable bacteria in capsules was found to be stable during storage. This allowed us to prepare FMT capsules ahead of time and they could be used at a moment's notice. This is an advantage over delivery through enema, which is commonly done at the FMT clinic in London, ON, as the process to prepare a fresh FMT takes up to one and a half hours and must be completed within an hour of the patient's scheduled visit. The administration of capsules is easier and more pleasant for delivery as patients are able to take the capsules themselves, avoiding an invasive procedure. The proven stability of the FMT capsules during storage has led to further FMT clinical trials being developed by our research group using capsules as the method of delivery of FMT.

It was found that different donors have different changes in viable bacteria during storage, and this phenomenon was noted by other studies of FMT material stability[7,8]. Differing changes in viable bacteria are likely a result of the unique inter-individual variation in the composition of the gut microbiome. While there were changes in the composition of viable bacteria, the total number of viable bacteria was comparable between donors during storage. If feasible in the future, clinics or stool banks could test each individual donor to determine how long their stool is stable for.

Seven of twenty-one NAFLD patients and two of five MS patients had abnormal intestinal permeability at baseline and all of these patients experienced improvement following FMT. This indicates that FMT may be beneficial for a subset of patients with NAFLD and MS. It also raises the question of whether increased intestinal permeability is responsible for causing and/or progressing disease in all patients? It is of interest that all three donors from the NAFLD-FMT clinical trial and both donors from the MS clinical trial were able to restore intestinal barrier function. Considering how difficult it was to find healthy donors

using the extended screening criteria that we developed[21], it was noteworthy that these parameters also selected for donors that could pass along the phenotype of normal intestinal barrier function in recipients that had abnormal permeability. Given that the numbers of patients with abnormal intestinal permeability and the number of donors were low in these studies, this theory should be tested in a larger number of patients with more FMT donors.

The liver is thought to be affected by increased intestinal permeability because it is the first organ, after the intestine, to encounter the blood supply, making it most vulnerable to antigens that have passed through the intestinal lumen as a result of impaired intestinal barrier function[22]. The innate immune system in the liver can be activated by these antigens and cause inflammation[23] leading to the development and progression of NAFLD[24]. If this clinical trial were to be repeated, a longer follow-up would be preferred to determine if PDFF improves after restoration of intestinal barrier function.

The progression of MS is slow and approximately half of patients with RRMS will progress to SPMS within ten years of diagnosis[25]. Thus, the benefits of normal small intestinal permeability on disease progression require longer time frames to properly assess. This study followed patients for a maximum of twelve months, and a meta-analysis of the average number of relapses per year in RRMS has shown that it can range from 0.27 to 1.66 [26], therefore it was unlikely for a patient in this study to experience a relapse during follow-up. A future study with longer follow-up that could potentially assess if the rate of progression of MS can be slowed with FMT is required to determine if improved intestinal barrier function is beneficial to MS patients.

Importantly, the results presented here represent the first study to show that intestinal permeability can be modulated with FMT. This has implications beyond the treatment of NAFLD and MS as a variety of conditions have been hypothesized to be caused or progressed by abnormal intestinal permeability, including rheumatoid arthritis[27], type 1 diabetes mellitus[28,29], and asthma[30].

This therapy would be ideal for diseases that have slow progression, like MS, as damage sustained from autoimmune diseases would not likely be repaired through FMT but could potentially be halted from progressing further. Therefore, FMT for MS would ideally be given shortly after diagnosis to retain gut barrier function and to alter the microbiota that may have caused disease onset. Another autoimmune disease that has slower progression is type 1 diabetes mellitus. This condition has a 'honeymoon' period, where the pancreas is still able to produce some insulin, and less than the predicted amount of administered insulin is required to control blood glucose levels[31]. Ideally, FMT could be given to type 1 diabetic patients at this stage to prolong the honeymoon period and slow the progression of the disease, but since ß islet cells cannot regenerate once they are lost[32], FMT would not restore full function of the pancreas. A rat model supports this hypothesis by showing that the increase in intestinal permeability occurs before the onset of type 1 diabetes[33]. Latent autoimmune diabetes in adults (LADA), which is a slower progressing form of autoimmune related diabetes[34], could be another potential application of FMT. At present it would be very difficult to predict who would go on to develop these diseases in humans and therefore it would not be practical to administer FMT prophylactically.

7.4 Strengths

7.4.1 Strengths of Model Organism Chosen

One of the strengths of this research was that we were able to study the diseases NAFLD and MS in the model that naturally develops the disease, humans. While it is likely that the sample sizes for these studies could have been larger if an animal model, for example mice, were used, they may not accurately reflect how humans would respond to FMT therapy.

Humans have distinct diets and gut physiology that differ from other animals that are commonly used as model organisms, and these differences can play a large role in the microorganisms that can survive and persist in the gut[35]. The gastrointestinal tract of different animal models can differ from humans in transit time, intestinal length, absorption, and pH[35,36]. Mice are most commonly used as the model organism for gut microbiome studies even though they are coprophagic, as well as being colonized by

microorganisms that are markedly different from humans[37]. Eighty-five percent of bacteria found in the gut of mice are not found in humans[38]. To counteract this, some studies use gnotobiotic mice or administer antibiotics, and give FMTs from human participants to try to humanize the gut microbiome of mice[39,40]. Humans are not germ-free and studying the gut microbiome in this model may not be relevant.

Obesity and the gut microbiome have been studied previously in mice as researchers found that an FMT from obese mice given to gnotobiotic mice caused significant weight gain and they believed that this was due to the increased energy harvesting potential of the obese gut microbiome[41]. A more recent study transplanted fecal matter from obese humans into gnotobiotic mice and there was significantly more weight gain observed than mice that received an FMT from the lean twins of the obese donor[39]. While the obese human donors had the same effect as the obese mice donors, these findings have never been replicated in human recipients. Studies have shown that the BMI of the FMT donor does not affect the BMI of a recipient post-transplant[42,43]. There has been one reported case of significant weight gain following FMT, but this weight gain was likely due to the recovery from rCDI, not FMT[44]. Mice do not normally develop NAFLD, but they can be induced to acquire the disease by a high-fat diet or genetic mutations that make mice resistant to leptin or produce non-functional leptin, so the sensation of satiety is suppressed[45]. Fibrosis can be developed in as early as six weeks when induced by diet[46] or less than 3 months of age when genetically induced[47]. While the mice develop a NAFLD like disease, given that it is induced using different mechanisms than in humans, it is not truly reflective of NAFLD in humans.

Similarly, mice do not naturally develop MS. There are two main models of MS-like disease in mice: experimental autoimmune encephalomyelitis (EAE) and Theiler's Murine Encephalitis Virus-Induced Demyelinating Disease (TMEV-IDD). EAE can be induced by immunizing mice with myelin antigens or by administering myelin-specific T cells, both of which result in demyelination in the brain[48]. While these methods successfully result in demyelination in the central nervous system, they do not induce the development of

demyelination through any mechanism in the gut, and therefore would not be relevant for an FMT study. Mouse models may not be appropriate for the study of NAFLD, MS and the gut microbiome, which is why a randomized control trial for humans was used instead.

7.4.2 Strengths of Techniques Utilized

One strength of this thesis was measuring changes in small intestinal permeability using the lactulose:mannitol urine test[49]. This test has been used as part of routine practice for over thirty years[50]. It is noninvasive, unlike assessing signs of increased intestinal permeability using histology on biopsies of the intestine[51]. The lactulose:mannitol test gives a quantitative output of intestinal permeability and is not subject to variation between histologists. Not all patients with increased intestinal permeability are detectable using histology[52], and therefore lactulose:mannitol test can be more sensitive than biopsy. This test was well-tolerated in patients and compliance was high, as all patients completed this test at home.

The use of PMA in combination with NGS was an additional strength of this study, as it limited the DNA from dead bacteria from being sequenced. PMA is able to bind to DNA from dead cells and free DNA, and therefore prevent its amplification during PCR and NGS[53]. Without PMA, this study would have been limited to identifying changes in viable bacteria in stool samples using culture-based techniques only. This would not have allowed the identification of what bacterial genera are most susceptible and resilient to the conditions that fecal samples are exposed to during preparation and storage before FMT.

7.5 Limitations

7.5.1 Limitations of FMT

Beyond infection risk, one limitation of using FMT as a microbiome therapy is that the long-term safety surrounding FMT is unknown as this treatment is relatively new. There may be microbes present in fecal matter that are not presently being screened for in donors that could unknowingly be passed onto FMT recipients and cause complications in the future. Common adverse events that are reported for FMT include: abdominal discomfort,

bloating, low-grade fever, flatulence, constipation, diarrhea, nausea, and vomiting[54]. More serious adverse events have been reported that include the transmission of infectious agents, such as drug-resistant *Escherichia coli*[55], bowel perforation[56], and aspiration during sedation for FMT delivered by colonoscopy[57]. The latter two serious adverse events were related to the method of delivery of FMT, not the FMT material itself. It is incumbent upon FMT clinics and stool banks to expand the list of exclusion criteria for potential donors to improve the safety surrounding FMT. Overall, there have been very few serious adverse events reported for FMT, but long-term follow-up has yet to occur[58].

One potential limitation of our clinical trials was that we were unable to match FMT recipients to donors by sex, as this has been shown to cause differences in the composition the gut microbiome[59]. During the NAFLD-FMT clinical trial, the only female donor moved out of London, ON and was no longer available to donate. We were unable to find a female replacement throughout the course of the study so only a male donor was used. A similar problem was encountered in the MS-FMT clinical trial where the only two donors who met the strict donor screening criteria were male. Females are more likely to have both metabolic syndrome[60] and MS[61] than males. Differences in the gut microbiota of humans have been found between females and males that have metabolic syndrome. There have been higher relative abundances in the genera of *Collinsella, Alistipes, Anaerotruncus,* and *Phascolarctobacterium* in females with metabolic syndrome compared to males with metabolic syndrome[62]. Differences in the gut microbiota composition between females and males has not been reported in MS. It is possible that an FMT that causes beneficial effects in one sex, may not have the same effect in another sex if a different composition of bacteria may be driving the disease in females versus males. Individuals with low, medium, or high serum concentrations of testosterone in males or low, medium or high serum concentrations of estradiol in females had differences in the relative abundance of several genera in the fecal bacterial composition[63]. There are some concerns that FMTs from a differing sex may result in hormonal changes in the recipient. It was always our intention to match donors and recipients by sex and this will be implemented in future studies. A larger number of

active donors would be needed to do this and samples from different sexes could be banked in the freezer to use at a later time.

At present, it is difficult to predict what FMT donors will go on to result in successful FMTs, and like organ transplants, compatibility between recipients and donors may be required. The lack of improvement in insulin resistance and PDFF in the NAFLD-FMT study may been due to incompatible donors, and in the future this may be overcome as compatibility factors are elucidated. On the other hand, some studies have identified what they call "super-donors" who have higher rates of success than their other FMT donors[64-66]. The reason for their high success rate has yet to be determined.

7.5.2 Limitations of Microbiome Analysis

A limitation of the toilet paper sampling method that was used to assess the fecal microbiota of patients that received FMT was that it did not assess changes in the microbiota composition of other areas of the gastrointestinal tract. The bacteria present on the toilet paper sample were likely more representative of the bacteria present on the mucosa in the distal colon, though this method is adequate to determine if there are major changes to the gut microbiota composition following FMT. There might have been changes in the composition of the gut microbiota in other regions of the GI tract as a result of FMT that were missed since they were not measured. However, taking samples of other sections of the GI tract would be costly and significantly more invasive for patients, like using an endoscopic brush to sample the small intestine[67], which could have negatively affected patient recruitment and retention for theses clinical trials.

We believe that the improvement in small intestinal permeability was in part due to increased production of butyrate by bacteria in the gut. One limitation of the two FMT clinical trials was that we did not measure of the concentration of SCFAs, such as butyrate, in the intestine of patients following FMT to determine if the improvement in small intestinal permeability was indeed correlated to its production by the gut microorganisms. Short-chain fatty acids in the gut were not measured because it would be an invasive

procedure for patients to sample the intestinal lumen in the small intestine. Previous studies on the concentrations of SCFAs in the intestine have been performed on patients during surgery or post-mortem during autopsy[68,69]. We did not measure SCFAs in stool samples because the concentration of SCFAs in stool differs from those found in different regions of the intestine[68] and the concentrations of SCFAs in stool do not necessarily relate to those at the sites of interest in the intestine. For example, butyrate can be overestimated if patients have shorter transit time, as there is less time butyrate to be absorbed by colonocytes before it exists the body[70].

If it were feasible to collect samples within the intestine, qPCR could also be used to determine the percentage of bacteria that possess the genes for butyrate production[71]. The proportion of butyrate producers could be examined to ascertain if the proportion of butyrate producers increased following FMT in patients that had improved small intestinal permeability. This technique could also be used on the stool samples that were collected from patients in both studies, however it is known that there are differences in the composition of bacteria in the small intestine versus stool[72]. The proportion of butyrate producers found in stool may not correlate with the proportion found in the small intestine.

In order to conduct 16S rRNA gene sequencing analysis, extracted DNA needs to be amplified with barcoded primers using PCR. Subsequently, a library is prepared for Illumina NGS, it is sequenced, and the data analyzed using bioinformatic tools. There is inherent bias in all steps of 16S rRNA gene sequencing[73]. The largest source of bias is the initial PCR step to amplify DNA before sequencing[73], as this occurs in the first step of library preparation and is carried on throughout the sequencing process. Universal primers for variable regions of the 16S rRNA gene can have primer bias and the 16S rRNA gene of different bacterial species can vary in amplification efficiencies[74]. This can lead to unequal amplification of different bacterial species and it can result in inaccurate relative abundances of bacteria being estimated during downstream analysis[74]. One way to overcome this would be to construct PCR-free Illumina libraries[73], but this was not feasible for our work as we needed barcoded primers to be able analyze multiple samples in one

Illumina sequencing run and the quantity of DNA in our samples was not large enough to bypass PCR. Using different thermocyclers and temperature ramp rates can also cause differences in library preparation for Illumina sequencing[73]. DNA sequences that have a G+C- and A+T-rich regions are amplified with lower efficiency and result in poorer quality library preparation for Illumina sequencing[75]. The same thermocycler and amplification protocol were used for all studies to eliminate variation. All of these sources of variation then contribute to bias when analyzing the 16S rRNA gene sequencing data using bioinformatics, as certain bacteria may be over or underrepresented in relative abundance.

Another limitation of the microbiota analysis conducted for the NALFD and MS clinical trials was that metagenomic sequencing and analysis was not performed due to cost and infrastructure required to handle the large data sets that would be produced. The microbiota analysis that was performed only resolved bacteria to the genus level, and not species or strain which can be done with whole genome shot-gun sequencing[76-77]. If the bacterial strain of each OTU was resolved, the identity of bacterial strains that belonged to the donor and if those specific strains were transferred to FMT recipients could be determined. The percentage of bacteria originating from the donor could be calculated following the FMT and the change in percentage of engraftment could be measured quantitatively post-FMT to help to establish how long an FMT is retained in recipients. Metagenomics could also be used to identify major biochemical pathways employed by the microbiota population and give insight into what metabolites are being produced. The price of whole genome shot-gun sequencing is continuing to decrease, and this technique will be utilized for future FMT clinical trials performed by our research group.

7.5.3 Limitations of Measuring Efficacy of FMT beyond rCDI

For a condition such as rCDI, the measurement of efficacy of FMT is binary: either there is prevention of further relapse or there is not. There is no consensus for measuring efficacy of FMT for conditions beyond rCDI. This becomes challenging for complex diseases that have a variety of causes, unlike rCDI which is caused by a single pathogen, and can be associated with different manifestations and symptoms between patients and within

patients over time. What if the FMT only improves one symptom of many that comprise a disease, should the FMT be considered effective? For the NAFLD-FMT clinical trial, there was improvement in small intestinal permeability, but not PDFF or insulin resistance. We believe that efficacy of FMT can be defined as an improvement in at least one disease outcome, as the possibility of seeing improvements in all indicators of a disease through FMT is unlikely. Engraftment of the donor may also need to be considered, however one study has shown that donor microbiota engraftment was not necessary for treating rCDI[78]. The same may not be true when FMT is used in other conditions.

7.6 The Future of FMT

The future of FMT therapy will likely include refinements to deliver the microorganisms or metabolites necessary for success without any unnecessary components of fecal matter. Until these components are identified, the way to best improve FMT may be to alleviate the burden of screening FMT donors. Being able to culture FMT material in the laboratory would be a benefit to FMT research, as one of the major limitations of establishing and running a stool bank is findings suitable donors[9-12,21,79]. In order to alleviate the need for human donors, a number of different methods could be used.

If the microbes necessary for success of FMT are limited, then a probiotic containing these microorganisms could be developed to supplement the missing microbes from the gut microbiome. Probiotics comprised of *Lactobacillus plantarum, Lactobacillus delbruekii, Lactobacillus acidophilus, Lactobacillus rhamnosus,* and *Bifidobacterium bifidum* have been used in patients with NASH and they resulted in a decrease in PDFF[80]. This provides justification that for the conditions of NAFLD and MS, which are complex and multifaceted, a mixture or consortia of bacteria may be required.

A product like SER-109, made by SERES Therapeutics that contains only bacterial spores has been reported to successfully treat rCDI[81], however this product is still donor derived and is not cultured *in vitro*. An example of a consortia that is grown in the lab is MET-1, previously "RePOOPulate", which contains 33 strains of commensal bacteria derived from

feces of a healthy human donor that can be grown *in vitro* in a chemostat model[82]. Growing up a fecal sample in a chemostat, an artificial gut model that can mimic the environment of different sections of the gastrointestinal tract, may prove beneficial since there would be no concern for transmission of disease since the bacteria are not grown in a human body. After the bacteria were isolated from a healthy donor, there would be no more need to recruit or screen healthy donors. This product would be defined and reproducible, unlike human feces, as the human gut microbiome composition is relatively stable, but it varies over time[83]. There are still limitations of culturing anaerobic bacteria *in vitro* and others have postulated that human derived FMT material could be cultured in the gut of an animal model, such as pigs, as they have been successfully colonized with human gut microbiomes[84]. Using an animal model could eliminate the need to screen for common human pathogens, as the model organisms are not hosts for these microbes. There are reservoirs of disease-free pigs that can be found in the sub-Antarctic Auckland Islands, isolated from human contact, that have been postulated to be suitable for *in vivo* cultivation of the human microbiome for FMT[35] as they have already been successfully used for islet transplantation in patients with type 1 diabetes[85].

7.7 Conclusions and Future Directions

There are a variety of methods currently in use to store and prepare fecal material for FMT, and this thesis has shown that there can be significant losses of bacteria as a result of these methods. While there does not appear to be an impact on the effectiveness of FMT for treating rCDI using FMT material prepared in a variety of ways, the expanded use of FMT for other conditions may be impacted by losses of vital microorganisms. Guidelines should be set in place for the storage and preparation of fecal material for FMT. Fecal samples should be stored at -80°C as whole stool or suspended in 10 % glycerol to result in the least amount of change in the composition and concentration of viable bacteria for stool samples prepared for delivery by enema, nasojejunal, endoscopy, or colonoscopy. FMT capsules are stable at -80 °C and can safely be stored long-term without significant decreases in viability or composition of bacteria. Both methods of preparation of FMT material would

likely benefit from anaerobic sample collection and processing and future work should be done to see if these methods improve bacterial viability. The optimized methods of storing and preparing fecal matter were used in both the NAFLD and MS clinical trials. FMT capsules are now being used for FMT clinical trials at the London, ON centre as they are able to be stored safely for long-term, they save time on the day of patients' appointments, they are easily administered, are lower in cost, and have increased appeal for recipients.

The donors selected using these expanded screening criteria were able to improve abnormal small intestinal permeability in both NAFLD and MS patients. It is widely hypothesized that improvements in abnormal intestinal permeability are a result of increased production of SCFAs, such as butyrate, by bacteria in the gut, which leads to an increase in tight junctions, and decreased paracellular transportation across the intestinal epithelium.

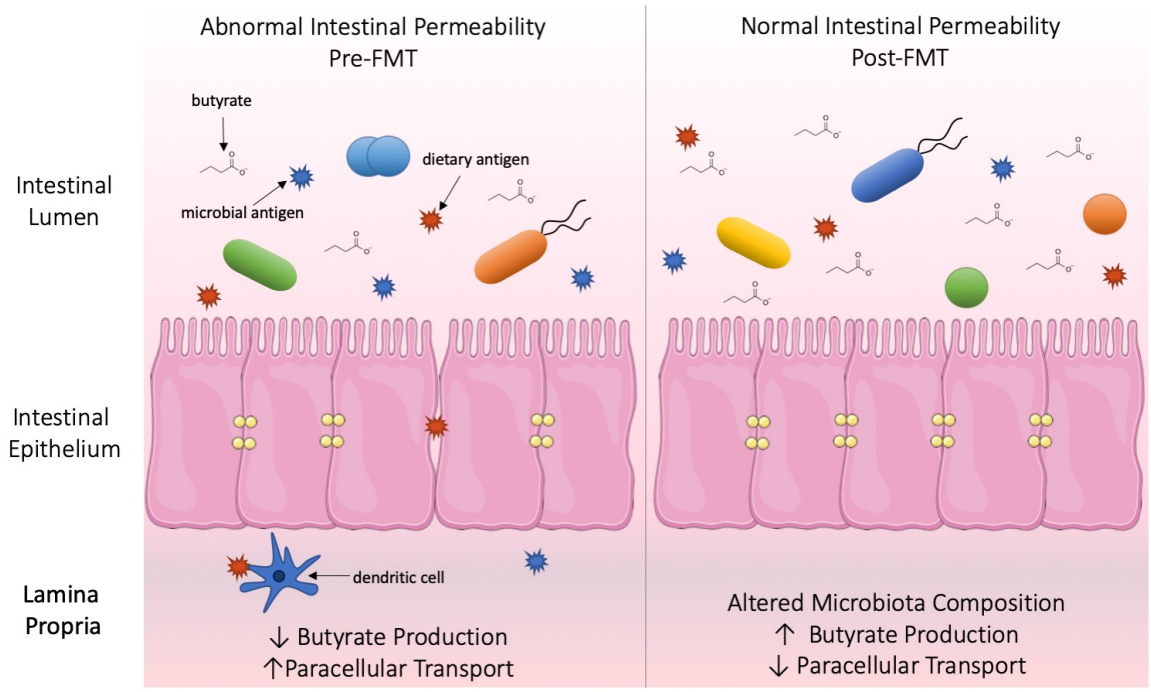

Figure 7.1 FMT restores normal intestinal permeability by providing butyrate-producing bacteria and increasing the prevalence of tight junction to limit paracellular transportation. The left side of the figure depicts abnormal intestinal permeability pre-FMT, where there is a decrease in butyrate producing microorganisms and a decrease in the number of tight junctions between intestinal epithelial cells. This leads to paracellular transport of microbial and dietary antigens across the intestinal epithelium into the lamina propria, which may contribute to the development and progression of MS and NAFLD through inflammation or activation of the immune system. Post-FMT there is an alteration in the microbial composition of the intestine, with an increase in the prevalence of butyrate producing bacteria leading to improved intestinal integrity, and microbial and dietary antigens are no longer able to pass paracellularly through the intestinal epithelium.

Further work is needed to determine how FMT is able alter intestinal permeability and what components of FMT are necessary for this effect. The fecal microbiota was shown to change at an individual level, but this work was not able to elucidate what bacteria may be

responsible for the improvement or cause of abnormal small intestinal permeability. Future work should be conducted using metagenomics to be able to assign taxonomy down to the species and/or strain level to determine what bacteria originated from the donor and the extent and duration of donor fecal engraftment in recipients and changes in genes encoding major metabolic pathways. While FMT was not shown to improve NAFLD or metabolic syndrome, the benefits of normal small intestinal permeability may take longer to manifest than the 6 months of follow-up performed in this study. It was also concluded that FMT in MS patients was safe and tolerable. Both NAFLD and MS are important diseases worldwide due their significant burden on the health care system and progression of symptoms that can significantly impact quality of life. While there are a number of therapies available for both diseases that seek to reduce the symptoms or treat disease, none are particularly efficient, and the prevalence of both of these diseases are still increasing. Future studies with larger sample sizes and longer durations of follow-up will be required to assess the efficacy of this intervention in the treatment of NAFLD and MS. Overall, this work provided evidence that the use of FMT can be expanded to other conditions that have been shown to have abnormal intestinal permeability, including both autoimmune and metabolic conditions, in the hope of slowing or halting the progression of these diseases.

7.8 References

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Appendix A – Chapter 2 Supplementary Material

Supplementary Table 2.1: Summary of statistics for total anaerobes at 4 °C for diluents over time

		Dunnett's Multiple Comparisons Test					
Diluent	**Ordinary one-way ANOVA**	**Fresh vs Blended**	**Fresh vs 4 h**	**Fresh vs 1d**	**Fresh vs 3d**	**Fresh vs 5d**	**Fresh vs 7d**
10 % Glycerol	**p=0.0036	p=0.4115	p=0.9151	p=0.5240	p=0.1292	p=0.1636	p=0.9102
Whole Stool	****p<0.0001	p<0.9999	p=0.9955	p=0.9997	***p=0.0002	**p=0.0013	*p=0.0121
Saline	***p=0.0002	p=0.9974	p=0.1328	p=0.3391	*p=0.0131	**p=0.0044	***p=0.0002
Water	**p=0.0027	*p=0.0187	p=0.1666	p=0.0881	p=0.9076	p=0.0532	p=0.1452

Supplementary Table 2.2: Summary of statistics comparing total anaerobes at 4 °C at each time point

		Tukey's Multiple Comparison Test					
Time	**Ordinary one-way ANOVA**	**10% Glycerol vs Whole Stool**	**10% Glycerol vs Saline**	**10% Glycerol vs Water**	**Whole stool vs Saline**	**Whole stool vs Water**	**Saline vs Water**
Blended	****p<0.0001	p=0.3955	p=0.6321	p=0.2596	p=0.9863	**p=0.0056	*p=0.0200
4 Hours	**p=0.0025	p=0.3629	p=0.3918	p=0.1130	*p=0.0231	**p=0.0028	p=0.9683
24 hours	*p=0.0315	p=0.6526	p=0.1943	*p=0.0230	p=0.8311	p=0.2949	p=0.7915
3 days	****p<0.0001	p=0.1143	p=0.9783	*p=0.0128	p=0.2475	****p<0.0001	**p=0.0045
5 days	p=0.8215						
7 days	p=0.0850						

Supplementary Table 2.3: Summary of statistics for Gram-positive anaerobes at 4 °C for diluents over time

		Dunnett's Multiple Comparisons Test					
Diluent	**Ordinary one-way ANOVA**	**Fresh vs Blended**	**Fresh vs 4 h**	**Fresh vs 1d**	**Fresh vs 3d**	**Fresh vs 5d**	**Fresh vs 7d**
10 % Glycerol	****p<0.0001	p=0.2496	p=0.9425	p=0.3941	****p<0.0001	***p=0.0006	p=0.2410
Whole Stool	****p<0.0001	p<0.9999	p=0.9998	p=0.1863	****p<0.0001	*p=0.0257	****p<0.0001
Saline	**p=0.0013	p=0.6919	p=0.1867	p=0.9996	**p=0.0033	**p=0.0059	p=0.0891
Water	p=0.1186						

Supplementary Table 2.4: Summary of statistics comparing Gram-positive anaerobes at 4 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
Blended	***p=0.0001	p=0.2403	p=0.9436	*p=0.0271	p=0.5447	****p<0.0001	**p=0.0055
4 Hours	**p=0.0010	p=0.8611	**p=0.0018	*p=0.0487	*p=0.0141	p=0.2168	p=0.7046
24 hours	**p=0.0018	p=0.9868	p=0.7019	**p=0.0065	p=0.5024	**p=0.0027	p=0.1084
3 days	p=0.6730						
5 days	p=0.7733						
7 days	p=0.1132						

Supplementary Table 2.5: Summary of statistics for lactobacilli at 4 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test					
		Fresh vs Blended	Fresh vs 4 h	Fresh vs 1d	Fresh vs 3d	Fresh vs 5d	Fresh vs 7d
10 % Glycerol	*p=0.0481	p=0.9627	p=0.9936	p=0.1661	p=0.7304	p>0.9999	p=0.9482
Whole Stool	****p<0.0001	p>0.9999	p=0.8981	p=0.2459	*p=0.0240	p=0.2728	****p<0.0001
Saline	**p=0.0012	**p=0.0044	p=0.2942	p>0.9999	p=0.9995	p=0.2389	p=0.4209
Water	****p<0.0001	p=0.9999	***p=0.0004	p=0.1097	****p<0.0001	*p=0.0141	*p=0.0315

Supplementary Table 2.6: Summary of statistics comparing lactobacilli at 4 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
Blended	**p=0.0024	p=0.8453	**p=0.0022	p=0.7921	*p=0.0244	p=0.9996	*p=0.0322
4 Hours	****p<0.0001	p=0.9824	p=0.2960	****p<0.0001	p=0.1629	****p<0.0001	*p=0.0155
24 hours	***p=0.0002	**p=0.0017	p=0.1911	p=0.9441	p=0.2885	***p=0.0005	p=0.0719
3 days	****p<0.0001	***p=0.0007	p=0.4868	****p<0.0001	*p=0.0476	****p<0.0001	****p<0.0001
5 days	****p<0.0001	p=0.3168	p=0.6337	*p=0.0112	*p=0.0239	****p<0.0001	p=0.1984
7 days	****p<0.0001	**p=0.0011	p=0.4990	*p=0.0208	****p<0.0001	****p<0.0001	p=0.4058

Supplementary Table 2.7: Summary of statistics for bifidobacteria at 4 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test					
		Fresh vs Blended	Fresh vs 4 h	Fresh vs 1d	Fresh vs 3d	Fresh vs 5d	Fresh vs 7d
10 % Glycerol	p=0.1383						
Whole Stool	**p=0.0022	p>0.9999	p=0.9738	p=0.8261	p=0.1189	p=0.6040	p=0.0924
Saline	****p<0.0001	p=0.1934	p=0.8057	p=0.2508	***p=0.0010	p=0.0609	**p=0.0042
Water	p=0.3703						

Supplementary Table 2.8: Summary of statistics comparing bifidobacteria at 4 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
Blended	****p<0.0001	*p=0.0448	***p=0.0003	****p<0.0001	p=0.3800	p=0.1647	p=0.9618
4 Hours	p=0.3809						
24 hours	p=0.4177						
3 days	**p=0.0081	p=0.2013	p=0.2281	p=0.6177	p=0.9999	*p=0.0197	*p=0.0232
5 days	p=0.1607						
7 days	**p=0.0090	*p=0.0101	*p=0.0275	p=0.2097	p=0.9837	p=0.5914	p=0.8058

Supplementary Table 2. 9: Summary of statistics for Gram-negative aerobes at 4 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test					
		Fresh vs Blended	Fresh vs 4 h	Fresh vs 1d	Fresh vs 3d	Fresh vs 5d	Fresh vs 7d
10 % Glycerol	****p<0.0001	**p=0.0086	****p<0.0001	p=0.1939	p=0.9347	p=0.8993	p=0.9997
Whole Stool	*p=0.0204	p<0.9999	p=0.2277	p=0.8267	p=0.2774	p=0.9464	p=0.5957
Saline	****p<0.0001	*p=0.0124	*p=0.0311	p=0.7478	p=0.0578	p=0.0822	p=0.2070
Water	****p<0.0001	*p=0.0498	****p<0.0001	p=0.5914	p=0.9999	p=0.9998	p=0.8671

Supplementary Table 2.10: Summary of statistics comparing Gram-negative aerobes at 4 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
Blended	***p=0.0001	***p=0.0004	p=0.8791	p=0.9998	**p=0.0044	***p=0.0005	p=0.9123
4 Hours	***p=0.006	*p=0.0366	p=0.1046	p=0.7168	p=0.9987	**p=0.0017	**p=0.0094
24 hours	p=0.1269						
3 days	p=0.1612						
5 days	p=0.2192						
7 days	p=0.1465						

Supplementary Table 2.11: Summary of statistics for Gram-positive aerobes at 4 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test					
		Fresh vs Blended	Fresh vs 4 h	Fresh vs 1d	Fresh vs 3d	Fresh vs 5d	Fresh vs 7d
10 % Glycerol	****p<0.0001	*p=0.0403	p=0.8170	* p=0.0122	p=0.5077	p=0.9651	** p=0.0013
Whole Stool	**p=0.0077	p>0.9999	p=0.4381	p=0.9997	p=0.7546	p=0.0736	p=0.9570
Saline	****p<0.0001	p=0.8719	p=0.1839	p=0.7936	**** p<0.0001	**** p<0.0001	**** p<0.0001
Water	*p=0.0311	p=0.6828	** p=0.0049	p=0.3214	p=0.2356	*p=0.031 0	p=0.1534

Supplementary Table 2.12: Summary of statistics comparing Gram-positive aerobes at 4 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
Blended	*** p=0.0001	*p=0.0165	**p=0.0021	***p=0.0001	p=0.9020	p=0.4348	p=0.836 2
4 Hours	**** p<0.0001	p<0.9999	p=0.1026	****p<0.000 1	p=0.0984	****p<0.000 1	p=0.159 2
24 hours	**** p<0.0001	*p=0.0253	**p=0.0024	****p<0.000 1	p=0.8536	p=0.1996	p=0.632 2
3 days	*** p=0.0005	p=0.7145	**p=0.0097	p=0.7343	***p=0.000 3	p=0.1676	p=0.128 9
5 days	*** p=0.0004	p=0.1195	***p=0.0006	**p=0.0023	p=0.2474	p=0.4226	p=0.991 7
7 days	**** p<0.0001	***p=0.000 4	****p<0.000 1	****p<0.000 1	**p=0.0075	p=0.2568	p=0.458 0

Supplementary Table 2.13: Summary of statistics for total anaerobes at -20 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10% Glycerol	**** p<0.0001	p=0.9944	p=0.9989	p=0.6457	p=0.1916	****p<0.0001
Whole Stool	**** p<0.0001	p=0.8996	p=0.0072	p=0.9298	p=0.4985	****p<0.0001
Saline	**** p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	**** p<0.0001	p=0.5038	*p=0.0476	***p=0.0006	****p<0.0001	****p<0.0001

Supplementary Table 2.14: Summary of statistics comparing total anaerobes at -20 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test: 10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	**** p<0.0001	p=0.9764	**** p<0.0001	p=0.4652	**p=0.0021	p=0.8055	*p=0.0113
2wk	**** p<0.0001	p=0.0760	*p=0.0211	p=0.0545	***p=0.0001	****p<0.0001	p=0.6615
4wk	**** p<0.0001	p=0.4363	**** p<0.0001	****p<0.0001	****p<0.0001	***p=0.0004	***p=0.0009
8wk	**** p<0.0001	p=0.9450	**** p<0.0001	***p=0.0002	****p<0.0001	****p<0.0001	p=0.5464
12wk	** p=0.0011	p=0.0727	*** p=0.0006	p=0.3227	p=0.5554	p=0.7672	p=0.0615

Supplementary Table 2.15: Summary of statistics for Gram-positive anaerobes at -20 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test: Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	****p<0.0001	p=0.3011	p=0.4812	p=0.8039	****p<0.0001	****p<0.0001
Whole Stool	****p<0.0001	p=0.8794	p>0.9999	p=0.9989	p=0.4609	****p<0.0001
Saline	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	****p<0.0001	p=0.9684	p=0.6447	**p=0.0089	**p=0.0032	****p<0.0001

Supplementary Table 2.16: Summary of statistics comparing Gram-positive anaerobes at -20 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test: 10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	**** p<0.0001	p=0.9011	** p=0.0034	p=0.8030	**p=0.0013	p=0.9994	***p=0.0002
2wk	p=0.1016						
4wk	**** p<0.0001	p=0.6342	**** p<0.0001	**p=0.0084	**** p<0.0001	** p=0.0010	**** p<0.0001
8wk	**** p<0.0001	****p<0.0001	p=0.9532	p=0.6607	**** p<0.0001	**p=0.0032	p=0.2320
12wk	**p=0.0034	p=0.9765	*p=0.0199	p=0.9204	p=0.1161	p=0.7624	**p=0.0031

Supplementary Table 2.17: Summary of statistics for lactobacilli at -20 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	p=0.5214					
Whole Stool	****p<0.0001	p=0.1010	**p=0.0031	*p=0.0371	***p=0.0006	****p<0.0001
Saline	****p<0.0001	p=0.4973	**p=0.0015	***p=0.0001	****p<0.0001	****p<0.0001
Water	p=0.3037					

Supplementary Table 2.18: Summary of statistics comparing lactobacilli at -20 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	*p=0.0388	*p=0.0466	p=0.2120	p=0.0781	p=0.8972	p=0.9964	p=0.9627
2wk	**p=0.0011	**p=0.0023	*p=0.0115	p=0.3781	p=0.9458	p=0.3005	p=0.2682
4wk	***p=0.0008	p=0.0628	***p=0.0003	p=0.0944	p=0.5594	p=0.9734	p=0.2203
8wk	****p<0.0001	p=0.0685	****p<0.0001	p=0.5176	**** p<0.0001	p=0.8343	***p=0.0001
12wk	****p<0.0001	****p<0.0001	***p=0.0002	p=0.1272	p=0.6413	**p=0.0050	p=0.1103

Supplementary Table 2.19: Summary of statistics for bifidobacteria at -20 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	****p<0.0001	p=0.8399	*p=0.0357	p=0.2767	****p<0.0001	****p<0.0001
Whole Stool	p=0.0624					
Saline	**p=0.0082	p=0.5843	p=0.5974	p=0.1364	**p=0.0057	**p=0.0044
Water	**p=0.0028	*p=0.0457	p=0.2696	p=0.0716	***p=0.0007	**p=0.0094

Supplementary Table 2.20: Summary of statistics comparing bifidobacteria at -20 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	p=0.1249						
2wk	p=0.9620						
4wk	p=0.3413						
8wk	**p=0.0049	*p=0.0464	p=0.6405	p=0.9663	p=0.1542	*p=0.0108	p=0.3097
12wk	p=0.1594						

Supplementary Table 2.21: Summary of statistics for Gram-negative aerobes at -20 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	**p=0.0044	p=0.2029	*p=0.0143	p=0.0715	*p=0.0276	p=0.9997
Whole Stool	****p<0.0001	p=0.8482	***p=0.0001	*p=0.0101	**p=0.0034	****p<0.0001
Saline	****p<0.0001	***p=0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	**p=0.0016	*p=0.0199	p=0.7788	*p=0.0106	**p=0.0030	*p=0.0396

Supplementary Table 2.22: Summary of statistics comparing Gram-negative aerobes at -20 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	****p<0.0001	*p=0.0199	****p<0.0001	**** p<0.0001	**** p<0.0001	p=0.2235	*p=0.0170
2wk	****p<0.0001	****p<0.0001	****p<0.0001	***p=0.0001	**** p<0.0001	p=0.2695	**** p<0.0001
4wk	****p<0.0001	****p<0.0001	****p<0.0001	**** p<0.0001	**** p<0.0001	p=0.5256	*p=0.0025
8wk	****p<0.0001	**p=0.0090	****p<0.0001	**p=0.0058	**** p<0.0001	p=0.7825	**p=0.0011
12wk	****p<0.0001	*p=0.0235	****p<0.0001	p=0.2603	*** p=0.0003	p=0.6492	**** p<0.0001

Supplementary Table 2.23: Summary of statistics for Gram-positive aerobes at -20 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	*p=0.0301	p=0.7903	p=0.9259	p=0.1500	p=0.9960	**p=0.0086
Whole Stool	**p=0.0074	p=0.9593	p=0.9844	p=0.0637	p=0.9839	p=0.1702
Saline	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	p=0.7748	****p<0.0001

Supplementary Table 2.24: Summary of statistics comparing Gram-positive aerobes at -20 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	****p<0.0001	p=0.2419	**p=0.0085	****p<0.0001	****p<0.0001	****p<0.0001	p=0.2380
2wk	****p<0.0001	p=0.6323	*p=0.0410	***p=0.0007	**p=0.0043	****p<0.0001	p=0.6424
4wk	**p=0.0037	p=0.9875	*p=0.0167	p=0.2947	**p=0.0073	p=0.1706	p=0.5789
8wk	****p<0.0001	p=0.9958	****p<0.0001	p=0.8510	****p<0.0001	p=0.6212	****p<0.0001
12wk	****p<0.0001	p=0.2582	p=0.3200	****p<0.0001	**p=0.0067	****p<0.0001	****p<0.0001

Supplementary Table 2.25: Summary of statistics for total anaerobes at -80 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	p=0.0570					
Whole Stool	*p=0.0158	p=0.2302	p=0.9864	p=0.7144	p=0.9421	p=0.4508
Saline	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	****p<0.0001	****p<0.0001	***p=0.0008	p=0.1239	***p=0.0006	****p<0.0001

Supplementary Table 2.26: Summary of statistics comparing total anaerobes at -80 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	**** p<0.0001	**** p<0.0001	p=0.9965	p=0.9962	****p<0.0001	****p<0.0001	p>0.9999
2wk	**** p<0.0001	**** p<0.0001	**p=0.0027	p=0.5949	****p<0.0001	****p<0.0001	***p=0.0003
4wk	**** p<0.0001	**** p<0.0001	p=0.6893	p=0.7612	****p<0.0001	****p<0.0001	p=0.9993
8wk	**** p<0.0001	p=0.4177	****p<0.0001	p>0.9999	****p<0.0001	p=0.4277	****p<0.0001
12wk	**** p<0.0001	**** p<0.0001	p=0.9430	p=0.7774	****p<0.0001	***p=0.0002	p=0.4721

Supplementary Table 2.27: Summary of statistics for Gram-positive anaerobes at -80 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 weeks	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	****p<0.0001	*p=0.0137	****p<0.0001	**p=0.0011	p=0.1241	****p<0.0001
Whole Stool	**p=0.0067	p=0.3311	p=0.7432	p=0.8421	p=0.9910	p=0.1458
Saline	****p<0.0001	p=0.2388	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	**p=0.0042	p=0.9999	p=0.4677	*p=0.0321	p=0.9006	**p=0.0050

Supplementary Table 2.28: Summary of statistics comparing Gram-positive anaerobes at -80 °C at each time point

Time	Ordinary One-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Waterl	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	*p=0.0131	**p=0.0099	p=0.8113	p=0.4718	p=0.0747	p=0.2487	p=0.9340
2wk	***p=0.0003	***p=0.0007	p=0.9190	p=0.4752	*p=0.0041	*p=0.0460	p=0.3527
4wk	**** p<0.0001	**p=0.0025	p=0.9112	p=0.9704	***p=0.0003	**p=0.0021	p=0.9989
8wk	**** p<0.0001	p=0.6538	*p=0.0222	p=0.9292	**** p<0.0001	p=0.9752	**p=0.0028
12wk	**p=0.0028	*p=0.0389	p=0.8911	p=0.1960	**p=0.0060	p=0.9477	*p=0.0494

Supplementary Table 2.29: Summary of statistics for lactobacilli at -80 °C for diluents over time

Diluent	Ordinary One-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 weeks	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	p=0.4776					
Whole Stool	****p<0.0001	p=0.2458	p=0.1902	***p=0.0009	****p<0.0001	****p<0.0001
Saline	****p<0.0001	p=0.9958	p=0.1927	p=0.9493	****p<0.0001	p=0.0018
Water	p=0.0622					

Supplementary Table 2.30: Summary of statistics comparing lactobacilli at -80 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	*p=0.0490	*p=0.0380	p=0.5299	p=0.9398	p=0.4229	p=0.1244	p=0.8608
2wk	*p=0.0247	p=0.0536	p=0.1003	p=0.9432	p=0.9605	p=0.2900	p=0.2863
4wk	***p=0.0001	****p<0.0001	*p=0.0369	*p=0.0169	p=0.1928	p=0.2240	p=0.9982
8wk	**p=0.0029	p=0.1044	p=0.0736	p=0.9204	p=0.9958	*p=0.0163	*p=0.0106
12wk	***p=0.0001	***p=0.0001	*p=0.0237	p=0.9787	p=0.1084	***p=0.0004	p=0.1274

Supplementary Table 2.31: Summary of statistics for bifidobacteria at -80 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	****p<0.0001	p=0.9671	p=0.1295	p=0.1951	p=0.8080	****p<0.0001
Whole Stool	****p<0.0001	****p<0.0001	****p<0.0001	p=0.9068	*p=0.0233	p=0.2439
Saline	****p<0.0001	p=0.9755	p=0.2283	**p=0.0017	****p<0.0001	****p<0.0001
Water	*p=0.0276	p=0.9997	p=0.4919	p=0.09911	p=0.2020	p=0.1081

Supplementary Table 2.32: Summary of statistics comparing bifidobacteria at -80 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	**** p<0.0001	**** p<0.0001	p=0.7733	p=0.9824	****p<0.0001	****p<0.0001	p=0.9356
2wk	**** p<0.0001	**** p<0.0001	p=0.9540	p<0.9999	****p<0.0001	****p<0.0001	p=0.9616
4wk	p=0.0549						
8wk	**** p<0.0001	***p=0.0010	**** p<0.0001	p=0.3252	****p<0.0001	****p<0.0001	**p=0.0072
12wk	**p=0.0028	p=0.1109	p=0.3836	p=0.3096	**p=0.0043	p=0.9573	*p=0.0178

Supplementary Table 2.33: Summary of statistics for Gram-negative aerobes at -80 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	p=0.7133	***p=0.0008
Whole Stool	p=0.1095					
Saline	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	**p=0.0100	p=0.9998	p=0.6263	p=0.5838	*p=0.0158	p=0.9794

Supplementary Table 2.34: Summary of statistics comparing Gram-negative aerobes at -80 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	**** p<0.0001	**** p<0.0001	****p<0.0001	***p=0.0001	p=0.2332	p=0.2324	**p=0.0016
2wk	**** p<0.0001	**** p<0.0001	****p<0.0001	*p=0.0150	p=0.2502	*p=0.0394	**p=0.0020
4wk	**** p<0.0001	**** p<0.0001	****p<0.0001	****p<0.0001	**p=0.0027	p=0.9813	**p=0.0024
8wk	**** p<0.0001	p=0.9531	****p<0.0001	p=0.0799	****p<0.0001	p=0.0747	**p=0.0051
12wk	**** p<0.0001	**** p<0.0001	****p<0.0001	**p=0.0013	*p=0.0180	*p=0.0315	****p<0.0001

Supplementary Table 2.35: Summary of statistics for Gram-positive aerobes at -80 °C for diluents over time

Diluent	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test				
		Fresh vs 1 week	Fresh vs 2 weeks	Fresh vs 4 weeks	Fresh vs 8 weeks	Fresh vs 12 weeks
10 % Glycerol	p=0.0570					
Whole Stool	*p=0.0158	p=0.2302	p=0.9864	p=0.7144	p=0.9421	p=0.4508
Saline	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001
Water	****p<0.0001	****p<0.0001	***p=0.0008	p=0.1239	***p=0.0006	****p<0.0001

Supplementary Table 2.36: Summary of statistics comparing Gram-positive aerobes at -80 °C at each time point

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test					
		10% Glycerol vs Whole Stool	10% Glycerol vs Saline	10% Glycerol vs Water	Whole stool vs Saline	Whole stool vs Water	Saline vs Water
1wk	****p<0.0001	p=0.0577	*p=0.0013	****p<0.0001	****p<0.0001	****p<0.0001	p=0.1811
2wk	***p=0.0006	p=0.3156	p=0.0748	p=0.0999	**p=0.0033	**p=0.0047	p=0.9995
4wk	p=0.0876						
8wk	****p<0.0001	p=0.7502	*p=0.0460	*p=0.0122	****p<0.0001	****p<0.0001	p=0.6011
12wk	****p<0.0001	*p=0.0216	p>0.9999	***p=0.0004	*p=0.0232	****p<0.0001	***p=0.0004

Appendix B – Chapter 3 Supplementary Material

Supplementary Table 3.1: Summary of statistics for viable total anaerobes throughout preparation and storage

		Dunnett's Multiple Comparisons Test							
Donor	**Ordinary one-way ANOVA**	**Fresh vs Capsule**	**Fresh vs 1 d**	**Fresh vs 3 d**	**Fresh vs 5 d**	**Fresh vs 1 wk**	**Fresh vs 2 wk**	**Fresh vs 1 m**	**Fresh vs 2 m**
1	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001
2	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001
3	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001

Supplementary Table 3.2: Summary of statistics comparing viable total anaerobes at each time point by donor

		Tukey's Multiple Comparison Test		
Time	**Ordinary one-way ANOVA**	**Donor 1 vs Donor 2**	**Donor 1 vs Donor 3**	**Donor 2 vs Donor 3**
Fresh	p=0.9778			
Capsule	p=0.1943			
1 d	****p<0.0001	p=0.9055	***p=0.0001	***p=0.0002
3 d	****p<0.0001	****p<0.0001	p=0.2234	****p<0.0001
5 d	p=0.6399			
1 wk	***p=0.0007	***p=0.0006	**p=0.0053	p=0.0834
2 wk	p=0.1862			
1 m	p=0.1774			
2 m	***p=0.0010	**p=0.0068	***p=0.0009	p=0.1167

Supplementary Table 3.3: Summary of statistics for viable Gram-positive anaerobes throughout preparation and storage

		Dunnett's Multiple Comparisons Test							
Donor	**Ordinary one-way ANOVA**	**Fresh vs Capsule**	**Fresh vs 1 d**	**Fresh vs 3 d**	**Fresh vs 5 d**	**Fresh vs 1 wk**	**Fresh vs 2 wk**	**Fresh vs 1 m**	**Fresh vs 2 m**
1	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001
2	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001
3	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001

Supplementary Table 3.4: Summary of statistics comparing viable Gram-positive anaerobes at each time point by donor

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test		
		Donor 1 vs Donor 2	Donor 1 vs Donor 3	Donor 2 vs Donor 3
Fresh	p=0.3125			
Capsule	p=0.3973			
1 d	*p=0.0141	p=0.9882	*p=0.0203	*p=0.0240
3 d	****p<0.0001	****p<0.0001	*p=0.0465	****p<0.0001
5 d	**p=0.0030	p=0.0654	**p=0.0024	*p=0.0484
1 wk	***p=0.0004	***p=0.0003	**p=0.0037	*p=0.0368
2 wk	***p=0.0005	***p=0.0008	p=0.9987	***p=0.0008
1 m	p=0.0514			
2 m	p=0.1145			

Supplementary Table 3.5: Summary of statistics for viable lactobacilli throughout preparation and storage

Donor	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test							
		Fresh vs Capsule	Fresh vs 1 d	Fresh vs 3 d	Fresh vs 5 d	Fresh vs 1 wk	Fresh vs 2 wk	Fresh vs 1 m	Fresh vs 2 m
1	**** p<0.0001	**** p<0.0001	*** p=0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	*** p=0.0001	*** p=0.0001	p=0.2483
2	*** p=0.0002	*** p=0.0002	*** p=0.0009	** p=0.0014	**** p<0.0001	**** p<0.0001	*** p=0.0001	**** p<0.0001	** p=0.0025
3	**** p<0.0001	**** p<0.0001	****p <0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001

Supplementary Table 3.6: Summary of statistics comparing viable lactobacilli at each time point by donor

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test		
		Donor 1 vs Donor 2	Donor 1 vs Donor 3	Donor 2 vs Donor 3
Fresh	*p=0.0127	*p=0.0170	p=0.9518	*p=0.0239
Capsule	*p=0.0369	*p=0.0310	p=0.2328	p=0.3110
1 d	****p<0.0001	****p<0.0001	p=0.4649	****p<0.0001
3 d	***p=0.0001	***p=0.0002	p=0.8493	***p=0.0002
5 d	****p<0.0001	****p<0.0001	****p<0.0001	**p=0.0043
1 wk	**p=0.0013	**p=0.0033	**p=0.0017	p=0.7300
2 wk	****p<0.0001	****p<0.0001	***p=0.0008	****p<0.0001
1 m	***p=0.0001	***p=0.0002	***p=0.0003	p=0.7188
2 m	**p=0.0027	**p=0.0068	**p=0.0036	p=0.5989

Supplementary Table 3.7: Summary of statistics for viable bifidobacteria throughout preparation and storage

		Dunnett's Multiple Comparisons Test							
Donor	Ordinary one-way ANOVA	Fresh vs Capsule	Fresh vs 1 d	Fresh vs 3 d	Fresh vs 5 d	Fresh vs 1 wk	Fresh vs 2 wk	Fresh vs 1 m	Fresh vs 2 m
1	*p=0.0180	p=0.8050	p=0.7008	p=0.2056	p=0.4880	p=0.0550	p=0.5015	p=0.8093	p=0.8093
2	**** p<0.0001	p=0.6683	p=0.5786	** p=0.0013	*p=0.0226	p=0.5330	p=0.9617	p=0.3633	p=0.7041
3	**** p<0.0001	**** p<0.0001	****p<0.0001	****p<0.0001	****p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001

Supplementary Table 3.8: Summary of statistics comparing viable bifidobacteria at each time point by donor

		Tukey's Multiple Comparison Test		
Time	Ordinary one-way ANOVA	Donor 1 vs Donor 2	Donor 1 vs Donor 3	Donor 2 vs Donor 3
Fresh	*p=0.0147	*p=0.0127	p=0.3367	p=0.0788
Capsule	**p=0.0079	**p=0.0084	*p=0.0230	p=0.6446
1 d	****p<0.0001	***p=0.0001	***p=0.0001	p=0.8792
3 d	****p<0.0001	***p=0.0002	***p=0.0001	p=0.8411
5 d	****p<0.0001	****p<0.0001	****p<0.0001	p=0.6634
1 wk	***p=0.0006	***p=0.0006	**p=0.0037	p=0.5205
2 wk	****p<0.0001	****p<0.0001	****p<0.0001	p=0.1321
1 m	**p=0.0010	****p<0.0001	***p=0.0006	p=0.1635
2 m	****p<0.0001	****p<0.0001	****p<0.0001	p=0.3993

Supplementary Table 3.9: Summary of statistics for viable Gram-negative aerobes throughout preparation and storage

		Dunnett's Multiple Comparisons Test							
Donor	Ordinary one-way ANOVA	Fresh vs Capsule	Fresh vs 1 d	Fresh vs 3 d	Fresh vs 5 d	Fresh vs 1 wk	Fresh vs 2 wk	Fresh vs 1 m	Fresh vs 2 m
1	** p=0.0037	p=0.6474	** p=0.0025	p=0.9999	p=0.7775	p=0.9142	* p=0.0370	p=0.8945	p>0.9999
2	** p=0.0056	*p=0.0362	p=0.1114	p=0.6923	** p=0.0076	*p=0.0152	** p=0.0055	** p=0.0064	** p=0.0025
3	**** p<0.0001	p=0.3978	p=0.9975	p=0.9999	p=0.9996	p=0.1549	**p=0.0011	*p=0.0223	p=0.1057

Supplementary Table 3.10: Summary of statistics comparing viable Gram-negative aerobes at each time point by donor

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test		
		Donor 1 vs Donor 2	Donor 1 vs Donor 3	Donor 2 vs Donor 3
Fresh	**p=0.0030	p=0.6642	**p=0.0083	**p=0.0035
Capsule	****p<0.0001	***p=0.0002	****p<0.0001	****p<0.0001
1 d	**p=0.0014	**p=0.0038	p=0.6478	**p=0.0017
3 d	****p<0.0001	p=0.1842	***p=0.0003	****p<0.0001
5 d	**p=0.0017	*p=0.0483	*p=0.0289	**p=0.0014
1 wk	***p=0.0005	**p=0.0068	*p=0.0278	***p=0.0004
2 wk	**p=0.0014	**p=0.0011	p=0.0576	*p=0.0182
1 m	**p=0.0043	*p=0.0191	p=0.3519	**p=0.0040
2 m	****p<0.0001	**p=0.0055	**p=0.0012	****p<0.0001

Supplementary Table 3.11: Summary of statistics for viable Gram-positive aerobes throughout preparation and storage

Donor	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test							
		Fresh vs Capsule	Fresh vs 1 d	Fresh vs 3 d	Fresh vs 5 d	Fresh vs 1 wk	Fresh vs 2 wk	Fresh vs 1 m	Fresh vs 2 m
1	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001	**** p<0.0001
2	**** p<0.0001	*** p=0.0005	** p=0.0011	*** p=0.0005	*** p=0.0007	*** p=0.0008	*** p=0.0009	** p=0.0014	** p=0.0011
3	*p=0.0189	*p=0.0121	*p=0.0106	**p=0.0094	*p=0.0110	**p=0.0065	**p=0.0065	**p=0.0074	**p=0.0065

Supplementary Table 3.12: Summary of statistics comparing viable Gram-positive aerobes at each time point by donor

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test		
		Donor 1 vs Donor 2	Donor 1 vs Donor 3	Donor 2 vs Donor 3
Fresh	*p=0.0275	*p=0.0369	p=0.9800	*p=0.0465
Capsule	****p<0.0001	****p<0.0001	p=0.9877	****p<0.0001
1 d	**p=0.0028	**p=0.0031	p=0.5569	**p=0.0087
3 d	*p=0.0212	*p=0.0191	p=0.4609	p=0.0883
5 d	**p=0.0040	**p=0.0039	p=0.4041	*p=0.0160
1 wk	**p=0.0011	**p=0.0015	p=0.7545	**p=0.0027
2 wk	**p=0.0010	**p=0.0017	p=0.9871	**p=0.0019
1 m	***p=0.0004	***p=0.0005	p=0.6428	**p=0.0011
2 m	**p=0.0011	**p=0.0013	p=0.6073	**p=0.0031

Supplementary Table 3.13: Summary of statistics for Shannon Diversity Index of viable bacteria throughout preparation and storage

Donor	Ordinary one-way ANOVA	Dunnett's Multiple Comparisons Test							
		Fresh vs Capsule	Fresh vs 1 d	Fresh vs 3 d	Fresh vs 5 d	Fresh vs 1 wk	Fresh vs 2 wk	Fresh vs 1 m	Fresh vs 2 m
1	** p=0.0022	*p=0.0103	p=0.9444	p=0.9952	p=0.8181	p=0.9328	p=0.9996	p=0.3777	NA
2	*** p=0.0003	*p=0.0121	**p=0.0024	p=0.0726	p=0.1202	*p=0.0126	***p=0.0007	***p=0.0004	****p<0.0001
3	p=0.1603						NA		

Supplementary Table 3.14: Summary of statistics comparing Shannon Diversity Index at each time point by donor

Time	Ordinary one-way ANOVA	Tukey's Multiple Comparison Test		
		Donor 1 vs Donor 2	Donor 1 vs Donor 3	Donor 2 vs Donor 3
Fresh	*p=0.0185	p=0.8765	*p=0.0220	*p=0.0394
Capsule	****p<0.0001	***p=0.0002	**p=0.0018	****p<0.0001
1 d	***p=0.0002	*p=0.0417	**p=0.0012	***p=0.0001
3 d	***p=0.0006	p=0.4390	**p=0.0020	***p=0.0007
5 d	****p<0.0001	p=0.8380	****p<0.0001	****p<0.0001
1 wk	***p=0.0006	p=0.5138	**p=0.0017	***p=0.0007
2 wk	NA	p=0.0772	NA	NA
1 m	****p<0.0001	p=0.0505	****p<0.0001	****p<0.0001
2 m	NA	NA	NA	***p=0.0001

Appendix C – Chapter 5 Supplementary Material

Supplementary Table 5.1 Summary of appointments and sample collection.

	Baseline	2 days	7 days	2 weeks	6 weeks	3 months	6 months
FMT	X						
Fecal Specimen	X						
MRI	X						X
Urine	X				X		
Diet History Questionnaire	X				X		
Blood	X			X	X		X
Toilet Paper	X	X	X	X	X	X	X

Supplementary Table 5.2 Summary of FMT donor characteristics.

	Donor 1	Donor 2	Donor 3
Age (years)	28	22	23
Sex	M	F	M
BMI	23.9	19.18	21.6
Hgb A1c (%)	5.2	5.2	4.8
Total Cholesterol (mmol/L)	4.71	4.22	4.25
HDL (mmol/L)	1.48	1.13	1.31
LDL (mmol/L)	2.98	2.14	3.2
Triglycerides (mmol/L)	0.54	2.09	1.62
ALT (U/L)	16	8	20
ALK phos (U/L)	27	111	80
Total bilirubin (µmol/L)	30.4	5	10
Creatinine (µmol/L)	79	59	89

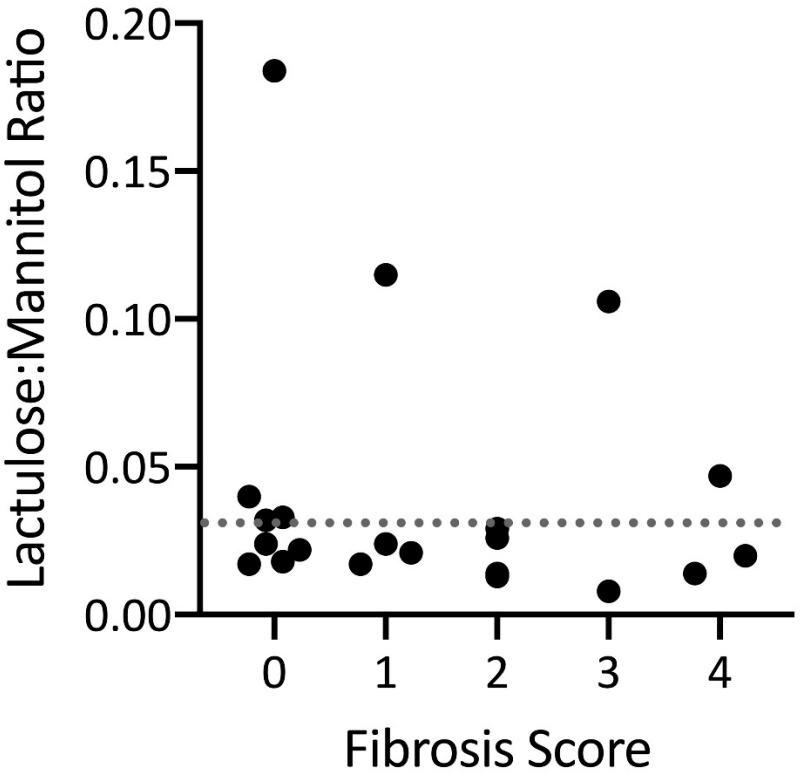
Supplementary Table 5.3 Histological scoring and hepatic PDFF of patients at baseline.

Patient	Treatment Group	Fibrosis Score	NAFLD Activity Score	Hepatic PDFF (baseline)
1	Allogenic	F4		31.457%
2	Allogenic	F0/1		32.908%
3	Allogenic	F1	4 (steatosis 2, lobular inflammation 1, ballooning 1)	21.287%
4	Allogenic	F3	2 (steatosis 1, lobular inflammation 1)	19.253%
5	Allogenic	F2		17.524%
6	Allogenic	F2		19.253%
7	Allogenic	F4		18.485%
8	Allogenic	F2	2 (steatosis 1, lobular inflammation 1)	15.008%
9	Allogenic	F3		11.971%
10	Allogenic	F1		11.509%
11	Allogenic	F4	3 (steatosis 1, lobular inflammation 1, ballooning 1)	15.941%
12	Allogenic	F0	1 (steatosis 1)	12.097%
13	Allogenic	F0/1		9.244%
14	Allogenic	F0/1		15.456%
15	Allogenic	F0/1		31.165%
16	Autologous	F2	3 (steatosis 2, lobular inflammation 1)	
17	Autologous	F0	2 (steatosis 1, inflammation 1)	32.378%
18	Autologous	F4	2 (steatosis 2)	2.219%
19	Autologous	F1	4 (steatosis 2, lobular inflammation 1, ballooning 1)	
20	Autologous	F0/1		28.379%
21	Autologous	F0/1		32.512%

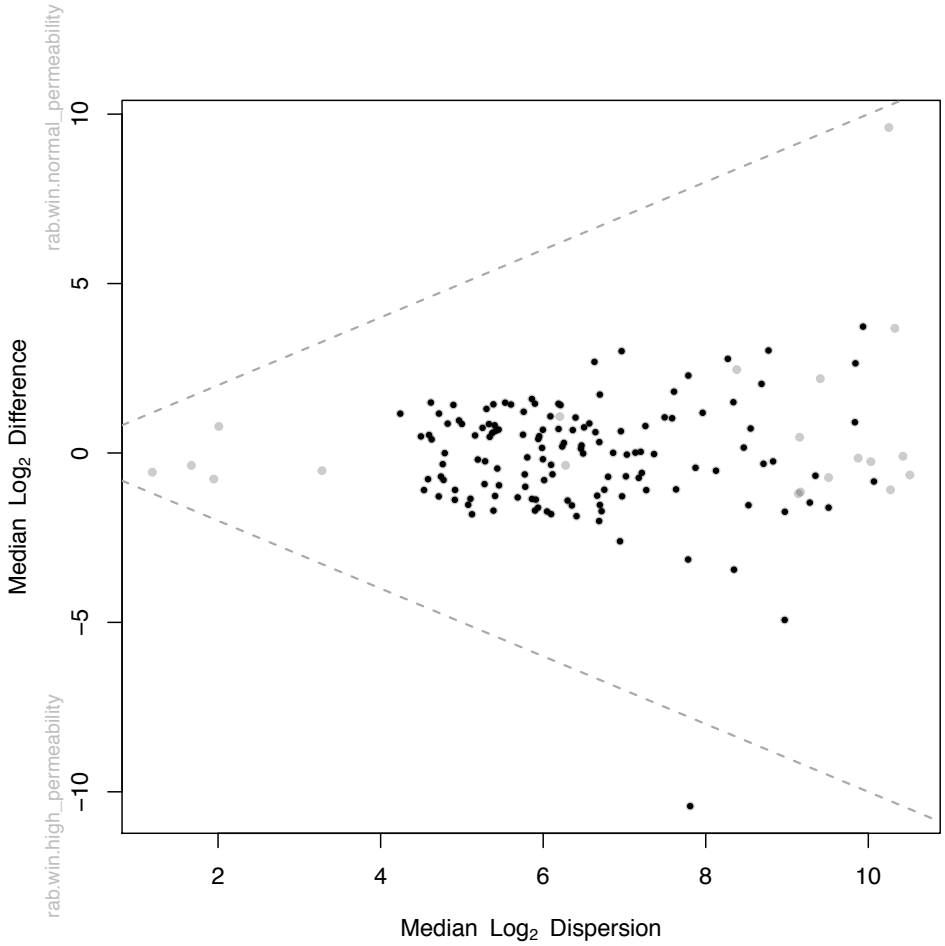
Nine patients had fibrosis staged by biopsy, seven patients were staged with fibroscan, and five patients were staged with MR elastography. All had steatosis documented by ultrasound.

NAFLD Activity Scores (NAS) are provided for those patients who underwent liver biopsy (25).

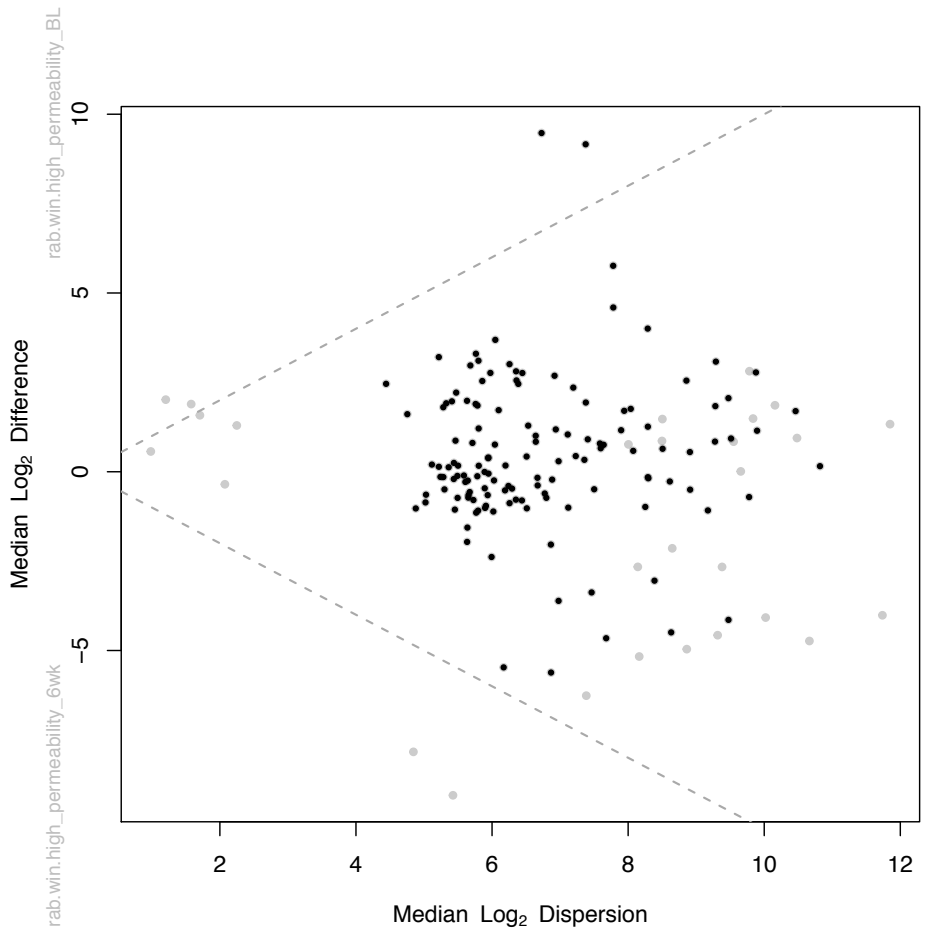
Two patients were unable to have an MRI.



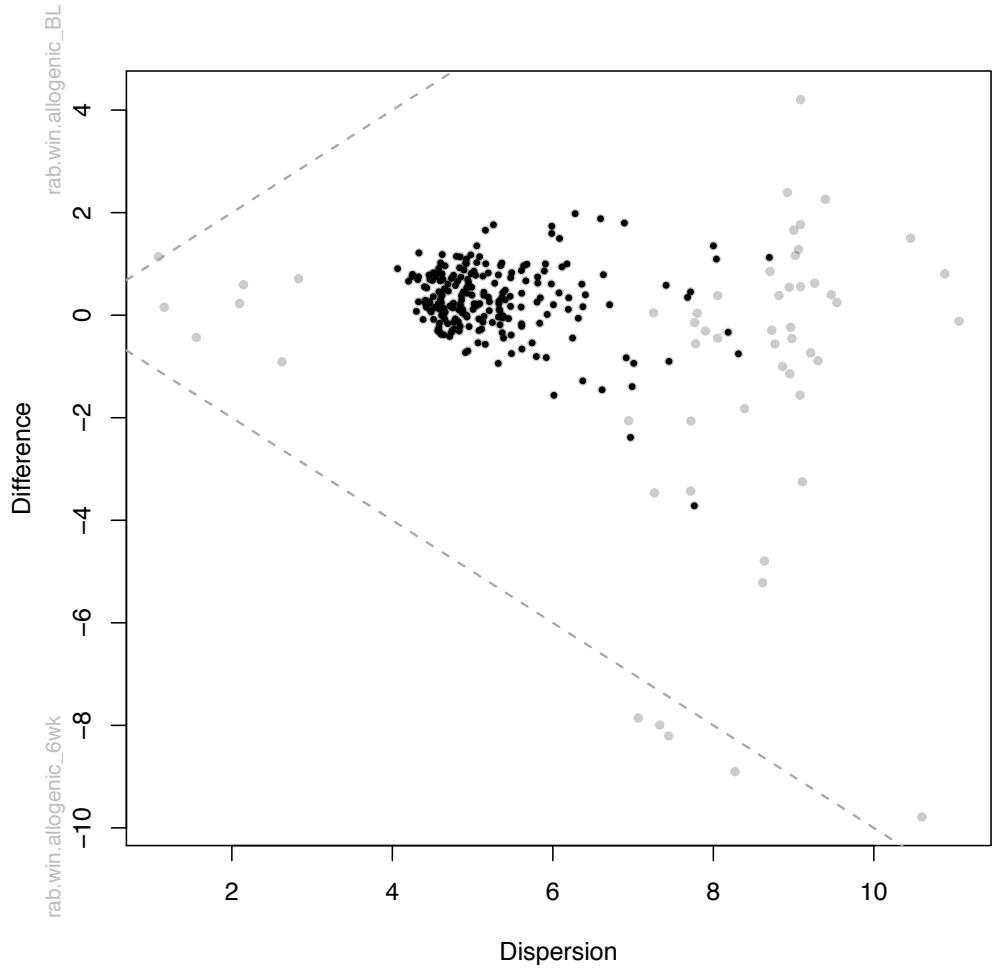
Supplementary Figure 5.1 Small intestinal permeability was not related to fibrosis score at baseline. Small intestinal permeability was determined using the lactulose:mannitol urine test. A ratio above 0.025 was considered to be abnormal. Fibrosis score (F0-F4) was determined by biopsy, fibroscan, or MR elastrography. Kruskal-Wallis test was used to compare the mean small intestinal permeability for each fibrosis score to one another, p=0.7767.



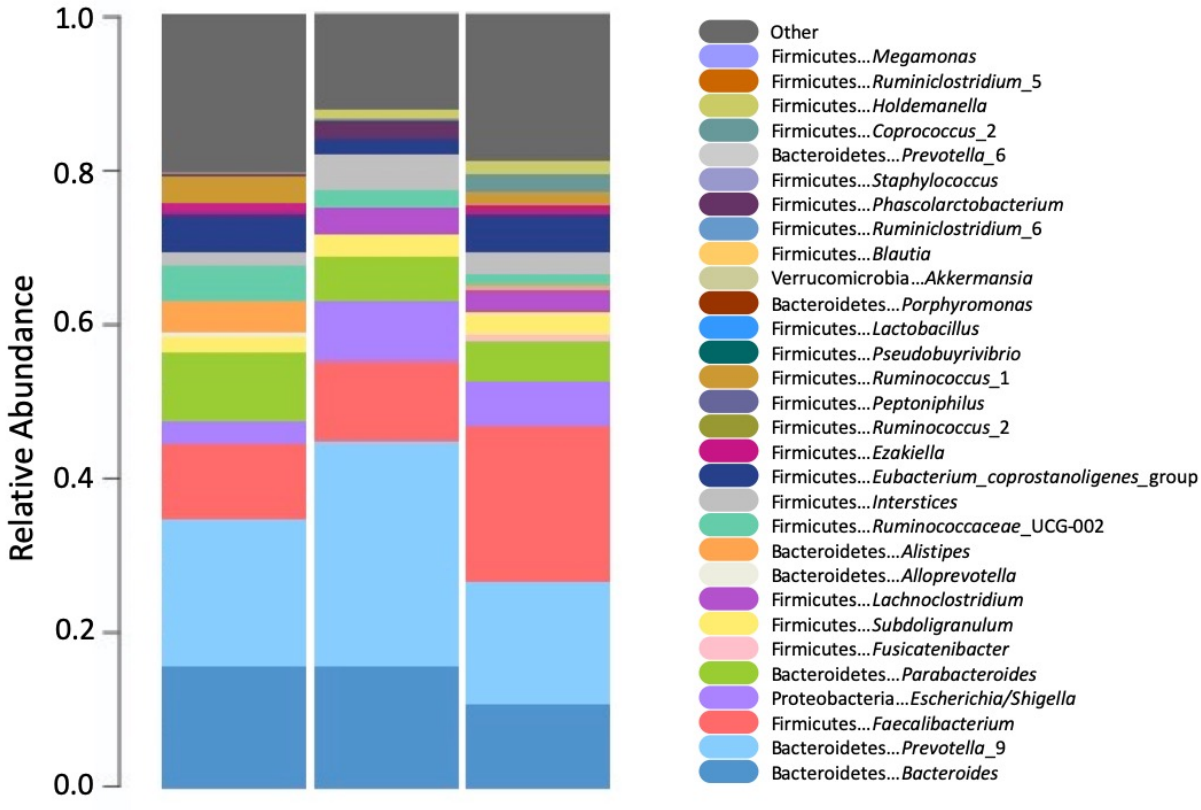
Supplementary Figure 5.2 Fecal microbiome comparison at baseline between allogenic FMT recipients with abnormally high small intestinal permeability versus patients with normal permeability. Fecal samples were collected by patients one day before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of NAFLD patients with abnormally high small intestinal permeability compared to NAFLD patients with normal small intestinal permeability at baseline. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red (effect size >|3|).



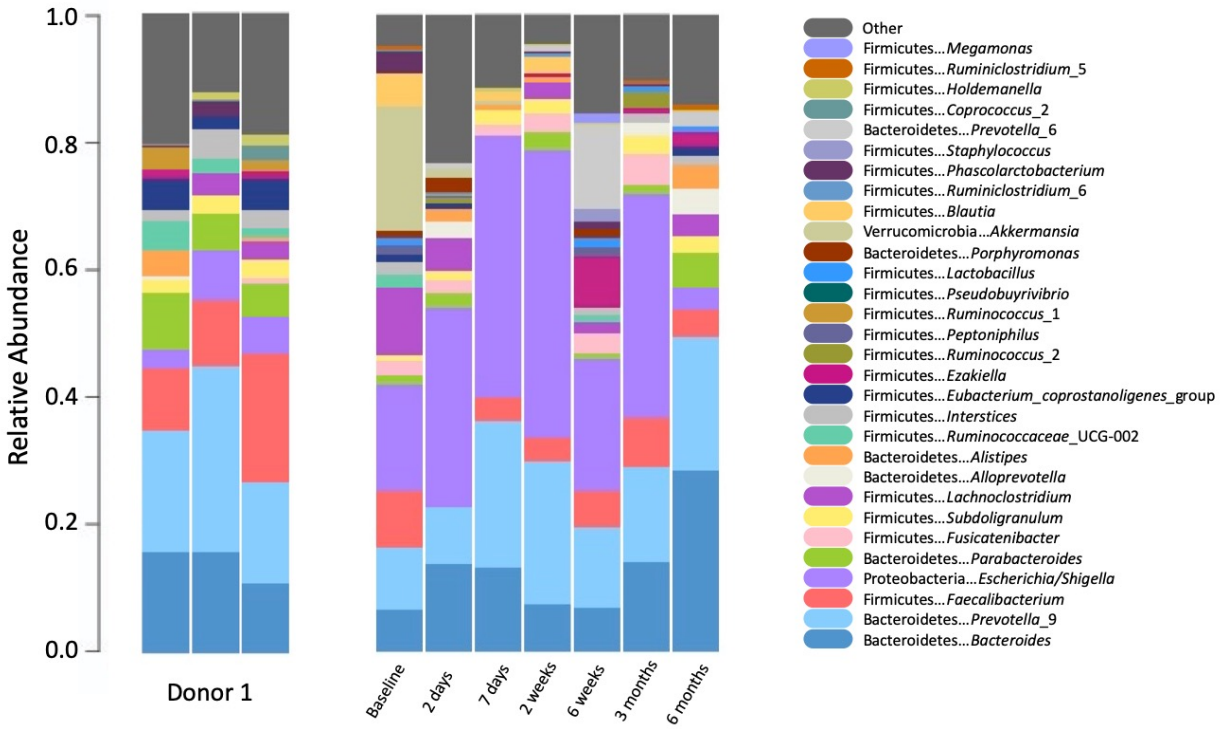
Supplementary Figure 5.3 Fecal microbiome comparison between baseline and 6 weeks in allogenic FMT recipients with abnormally high small intestinal permeability. Fecal samples were collected by patients one day before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of NAFLD patients with abnormally high permeability at baseline compared to 6 weeks following the FMT. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red (effect size >|3|).



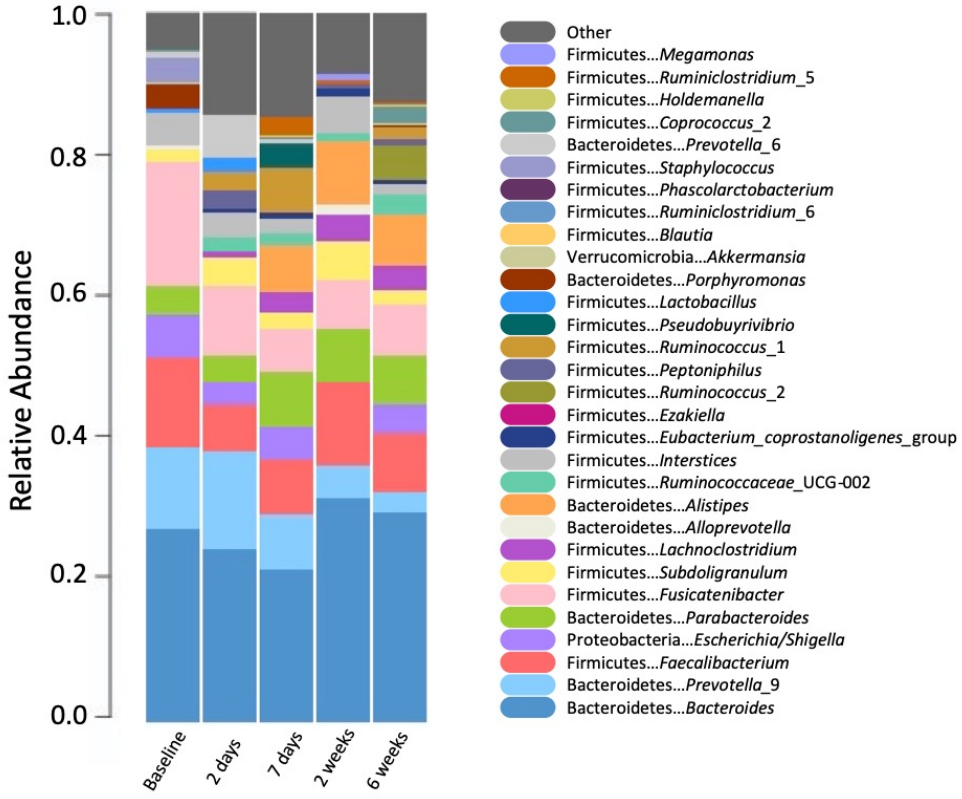
Supplementary Figure 5.4 Fecal microbiome comparison between baseline and 6 weeks in allogenic FMT recipients. Fecal samples were collected by patients one day before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of NAFLD patients with abnormally high permeability at baseline compared to 6 weeks following the FMT. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red (effect size >|3|).



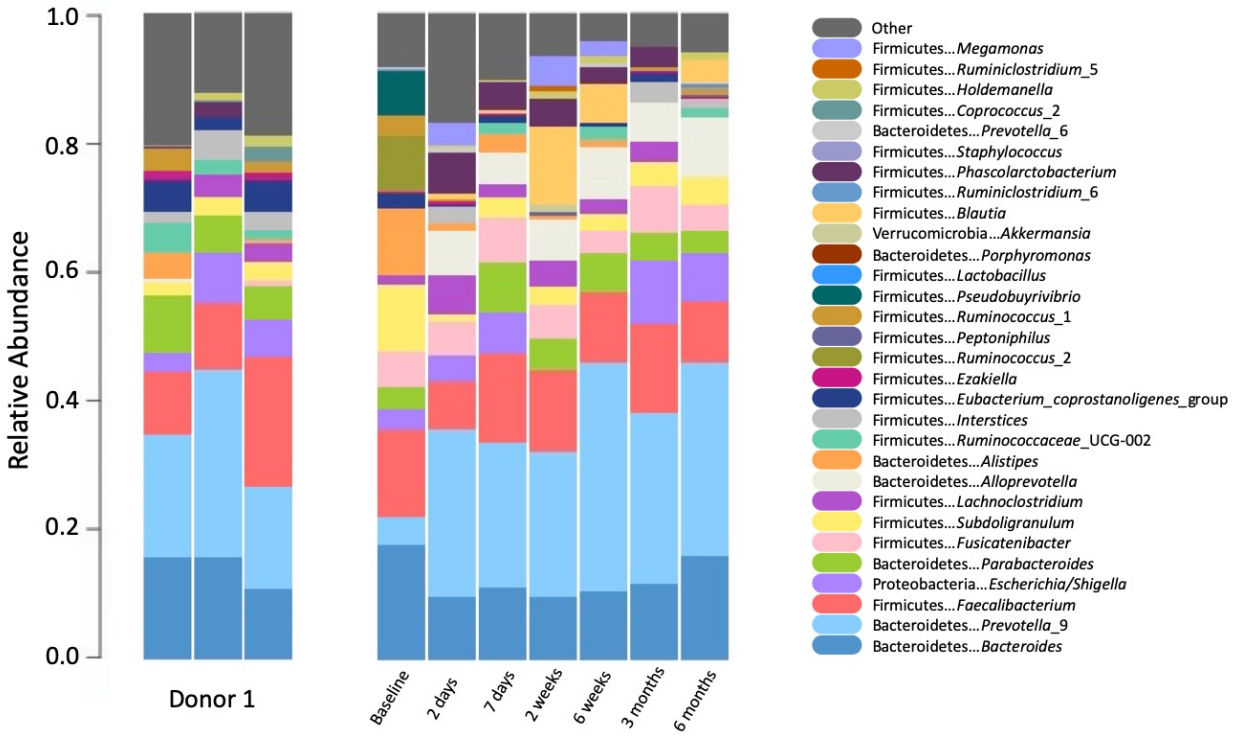
Supplementary Figure 5.5 Fecal microbiota composition of Donor 1 throughout the clinical trial. Toilet paper samples were collected from the donor every three months throughout the study. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



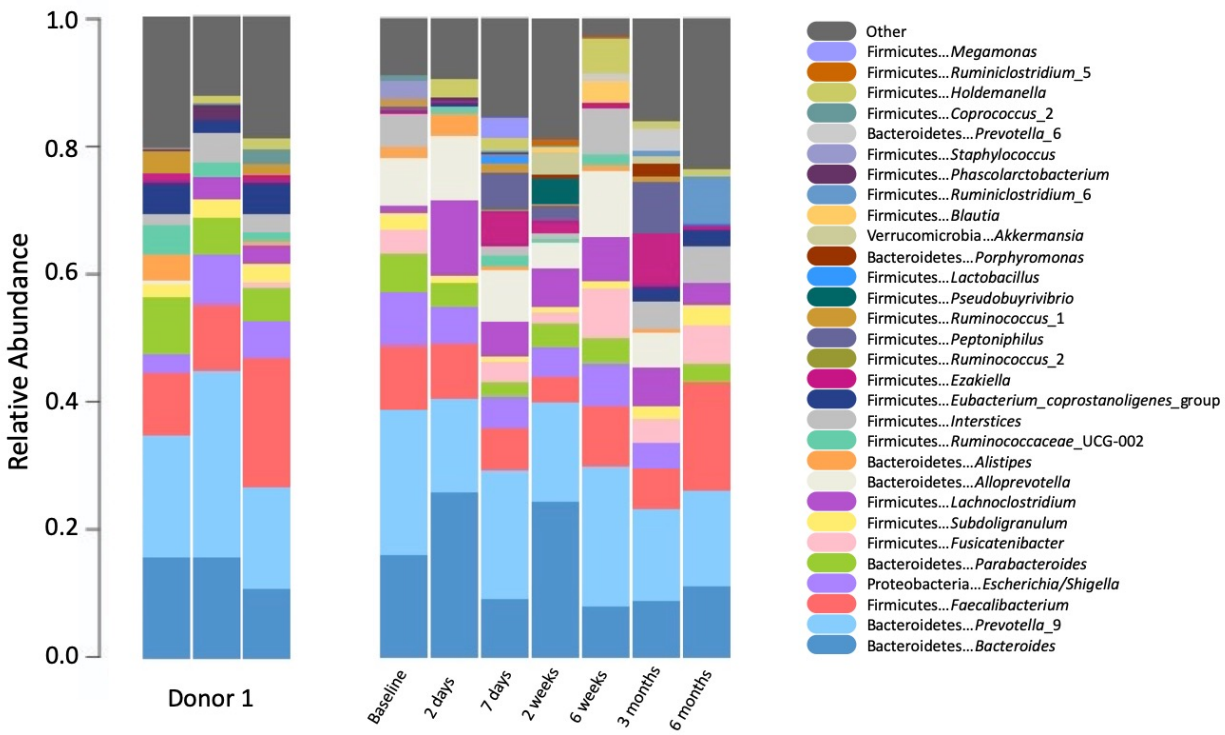
Supplementary Figure 5.6 Fecal microbiota composition of Patient 1 following an allogenic FMT. Patient 1 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



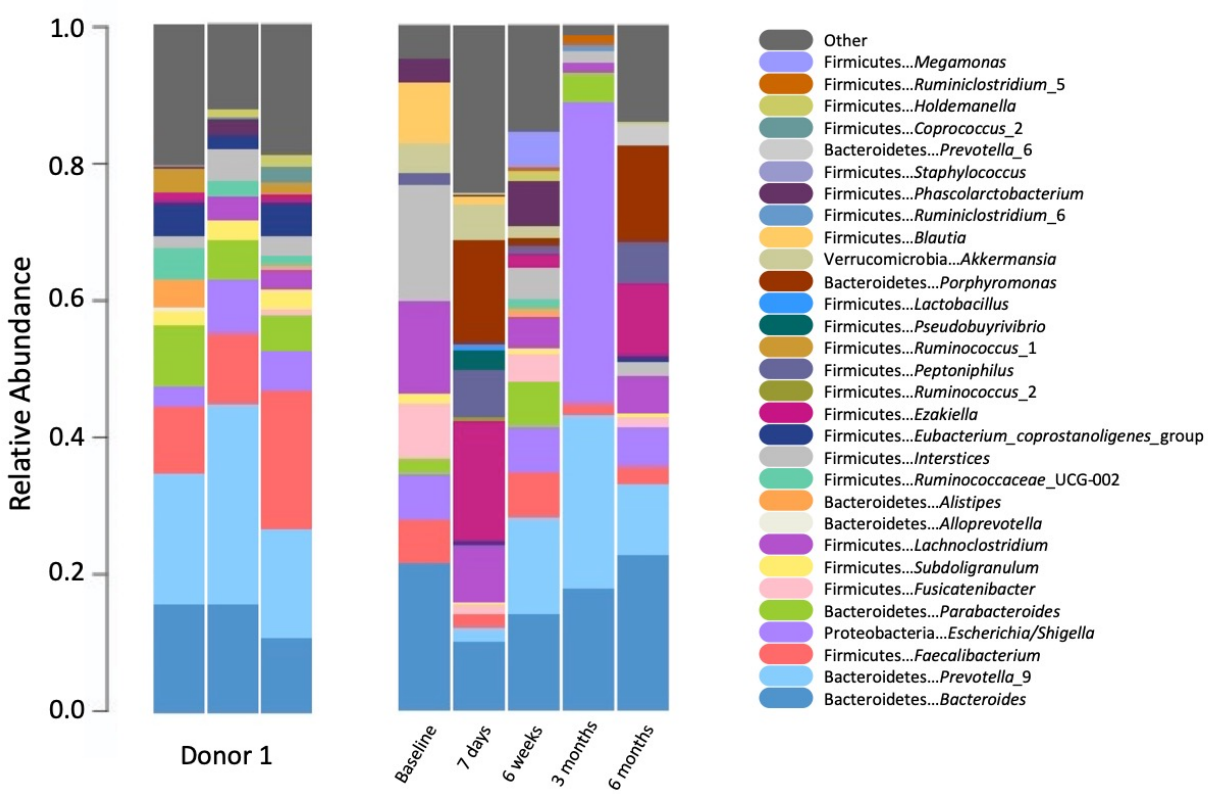
Supplementary Figure 5.7 Fecal microbiota composition of Patient 2 following an allogenic FMT. Patient 2 received an allogenic FMT from Donor 3. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



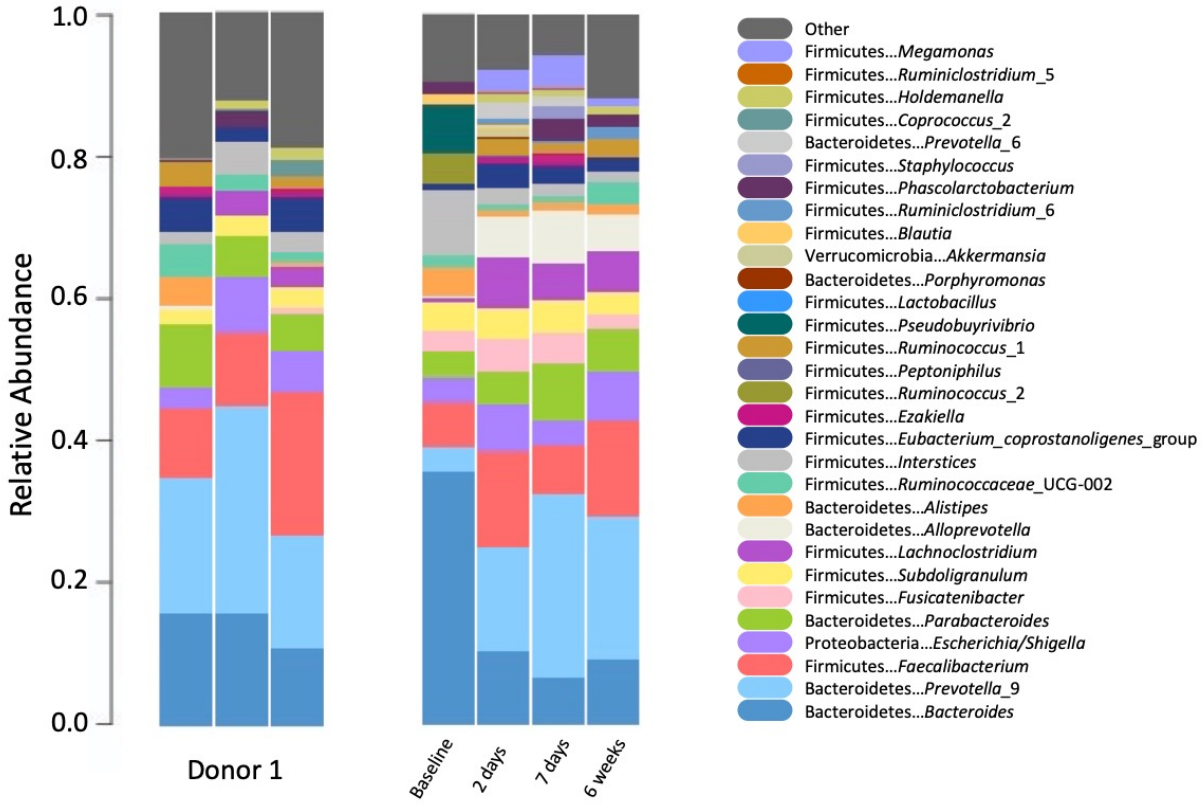
Supplementary Figure 5.8 Fecal microbiota composition of Patient 3 following an allogenic FMT. Patient 3 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



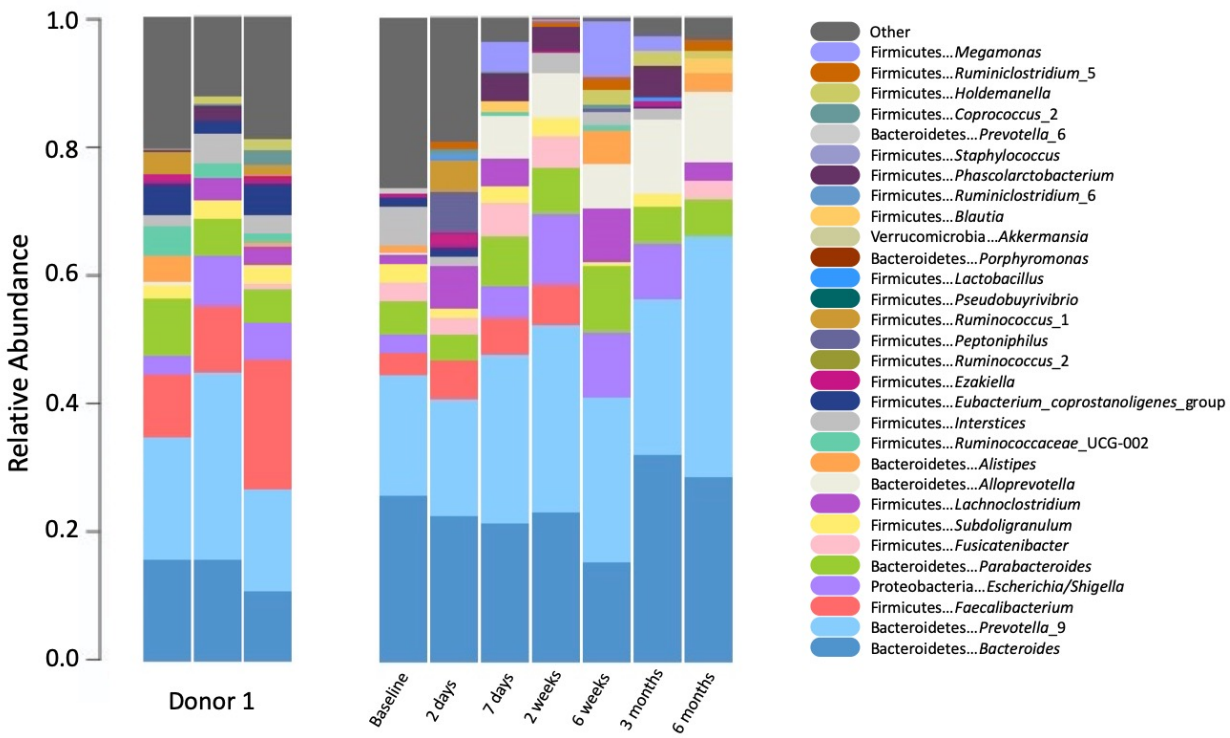
Supplementary Figure 5.9 Fecal microbiota composition of Patient 4 following allogenic FMT. Patient 4 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



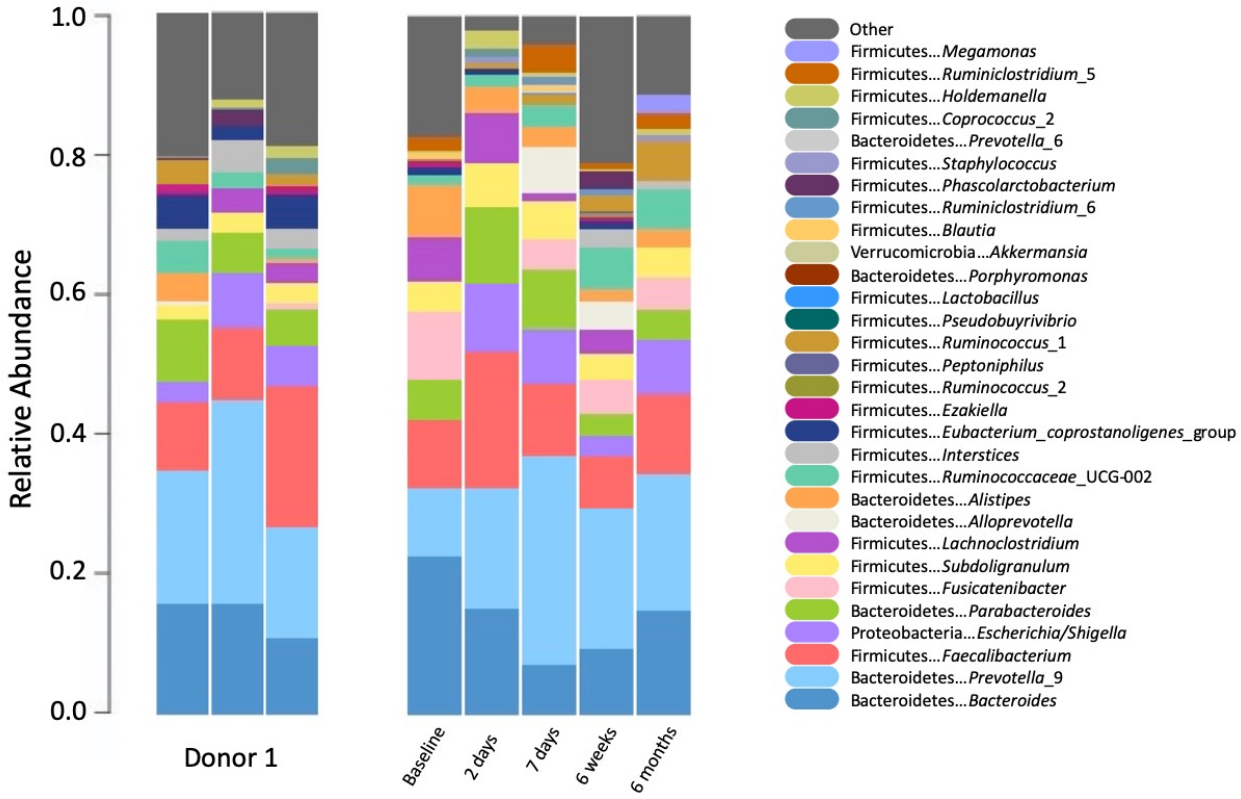
Supplementary Figure 5.10 Fecal microbiota composition of Patient 5 following allogenic FMT. Patient 5 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



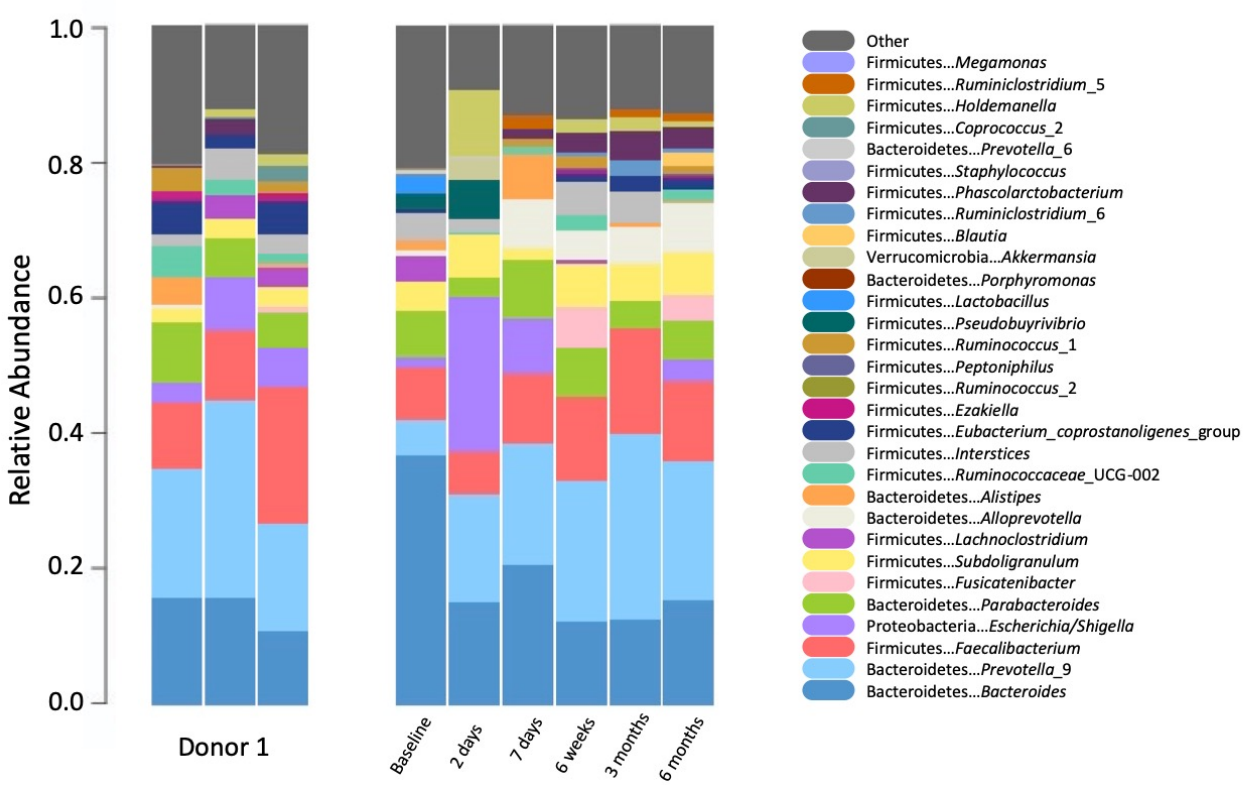
Supplementary Figure 5.11 Fecal microbiota composition of Patient 6 following an allogenic FMT. Patient 6 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



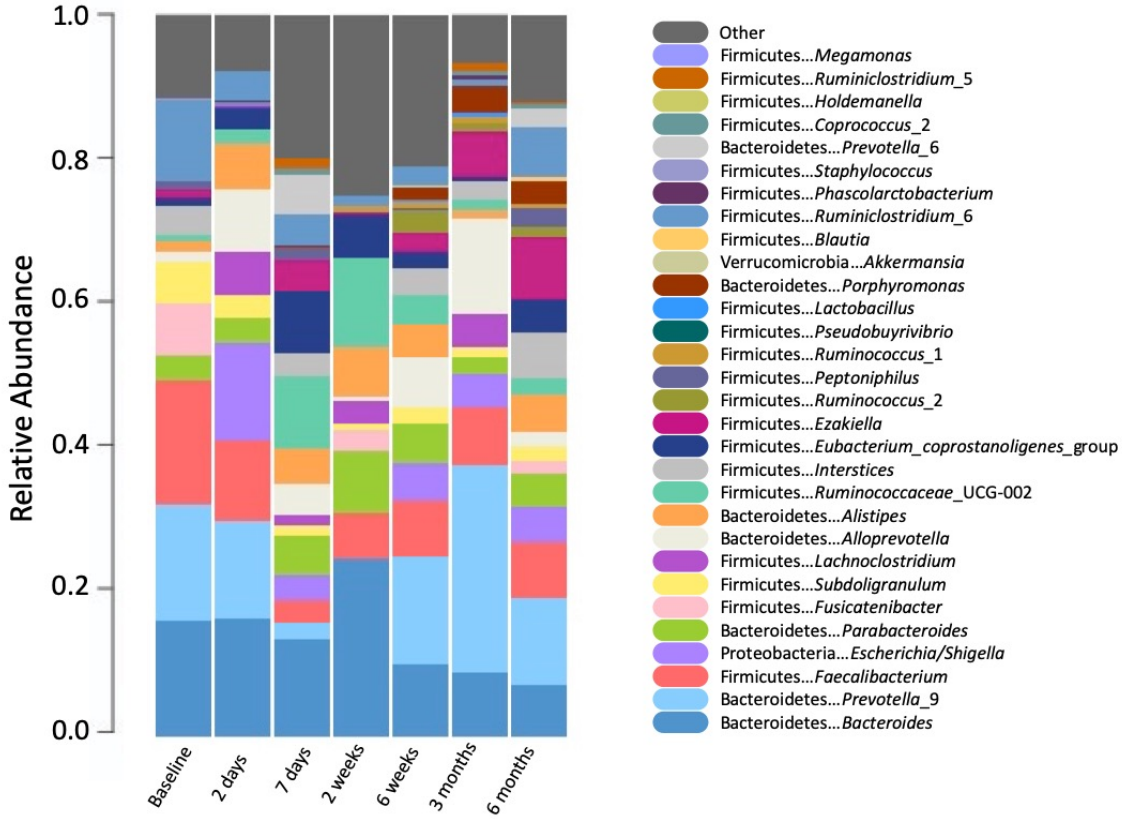
Supplementary Figure 5.12 Fecal microbiota composition of Patient 7 following an allogenic FMT. Patient 7 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



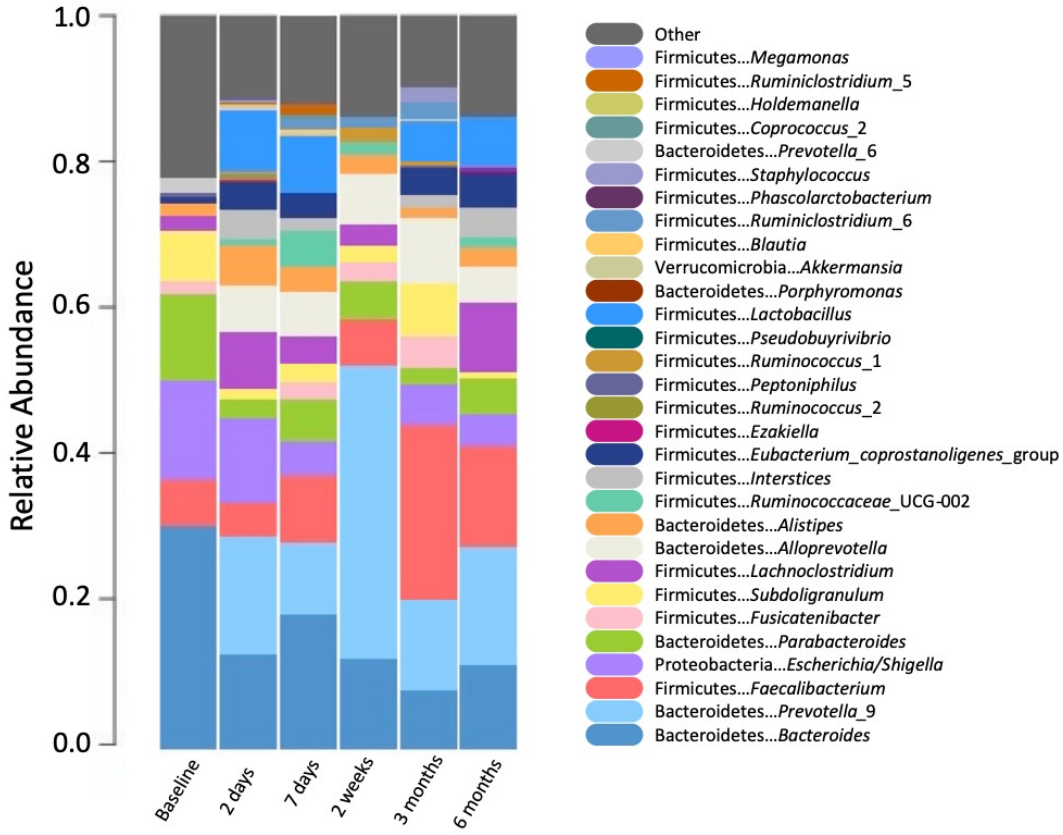
Supplementary Figure 5.13 Fecal microbiota composition of Patient 8 following an allogenic FMT. Patient 8 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



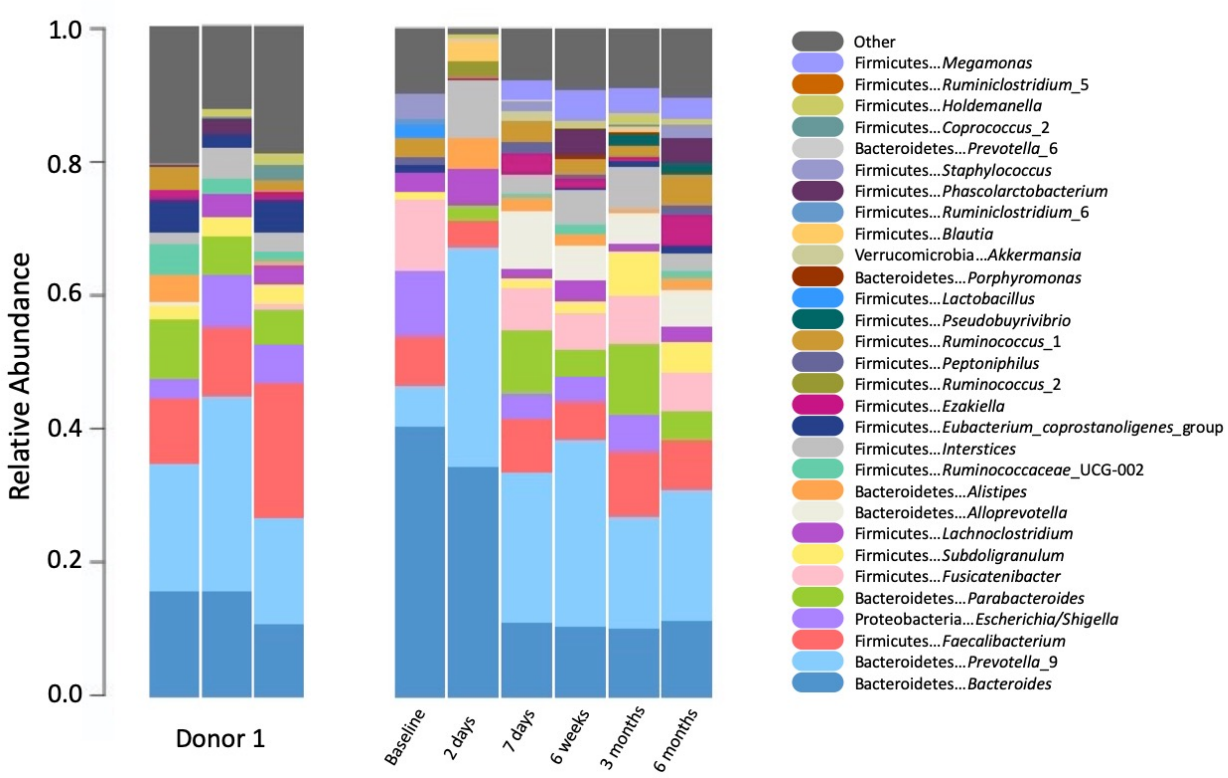
Supplementary Figure 5.14 Fecal microbiota composition of Patient 9 following an allogenic FMT. Patient 9 received an allogenic FMT from Donor 1. Toilet paper samples were collected from the patient at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



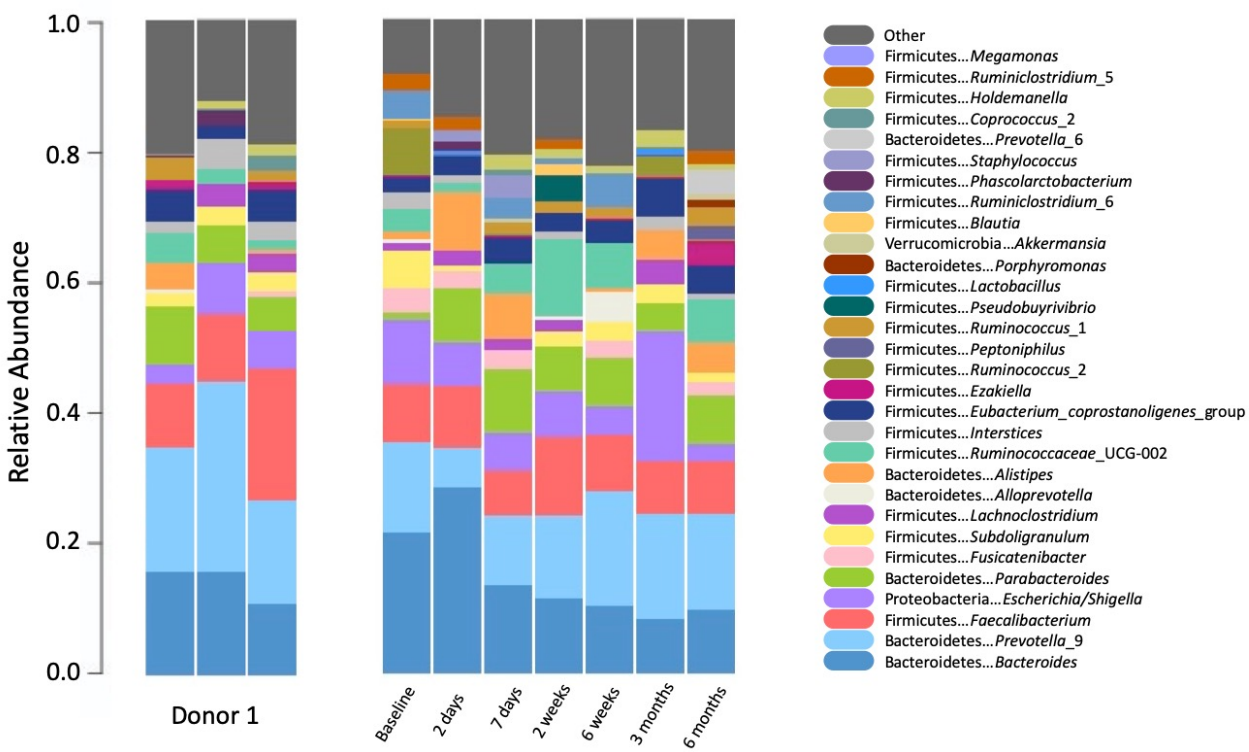
Supplementary Figure 5.15 Fecal microbiota composition of Patient 10 following an allogenic FMT. Patient 10 received an allogenic FMT from Donor 2. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



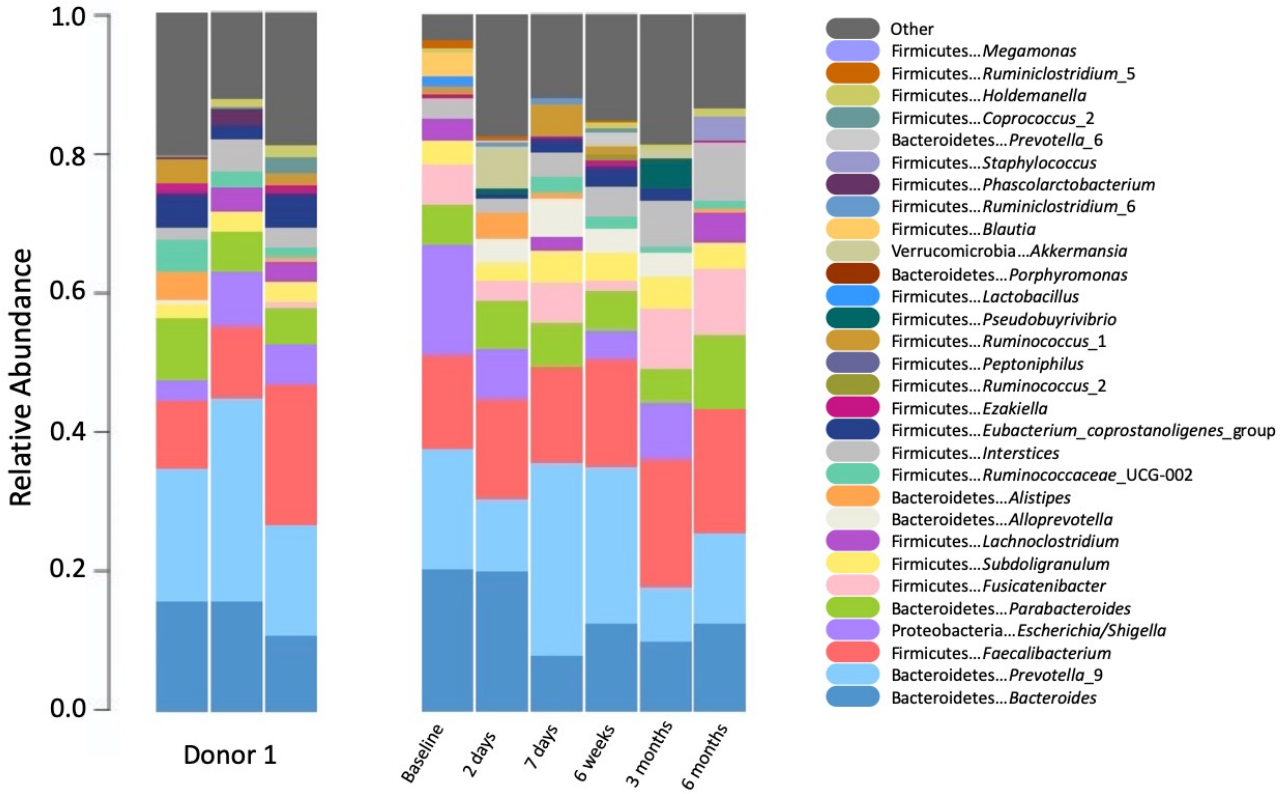
Supplementary Figure 5.16 Fecal microbiota composition of Patient 11 following an allogenic FMT. Patient 11 received an allogenic FMT from Donor 2. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



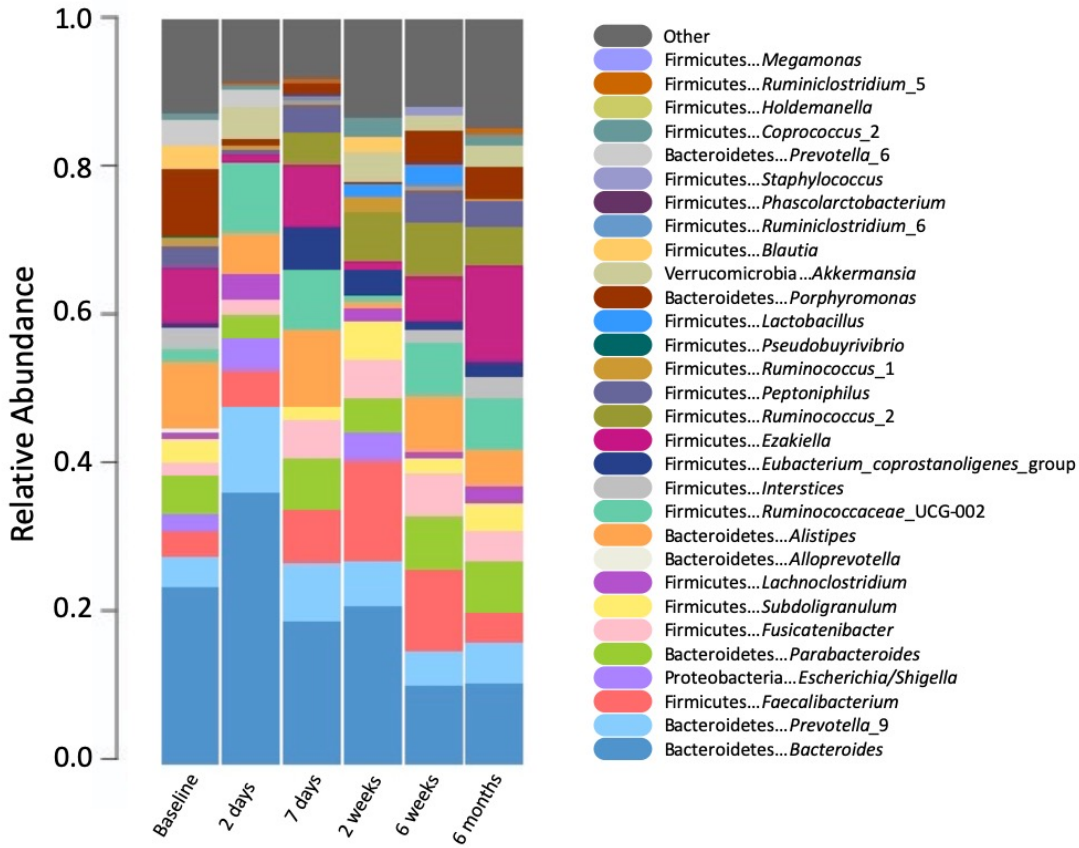
Supplementary Figure 5.17 Fecal microbiota composition of Patient 12 following an allogenic FMT. Patient 12 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



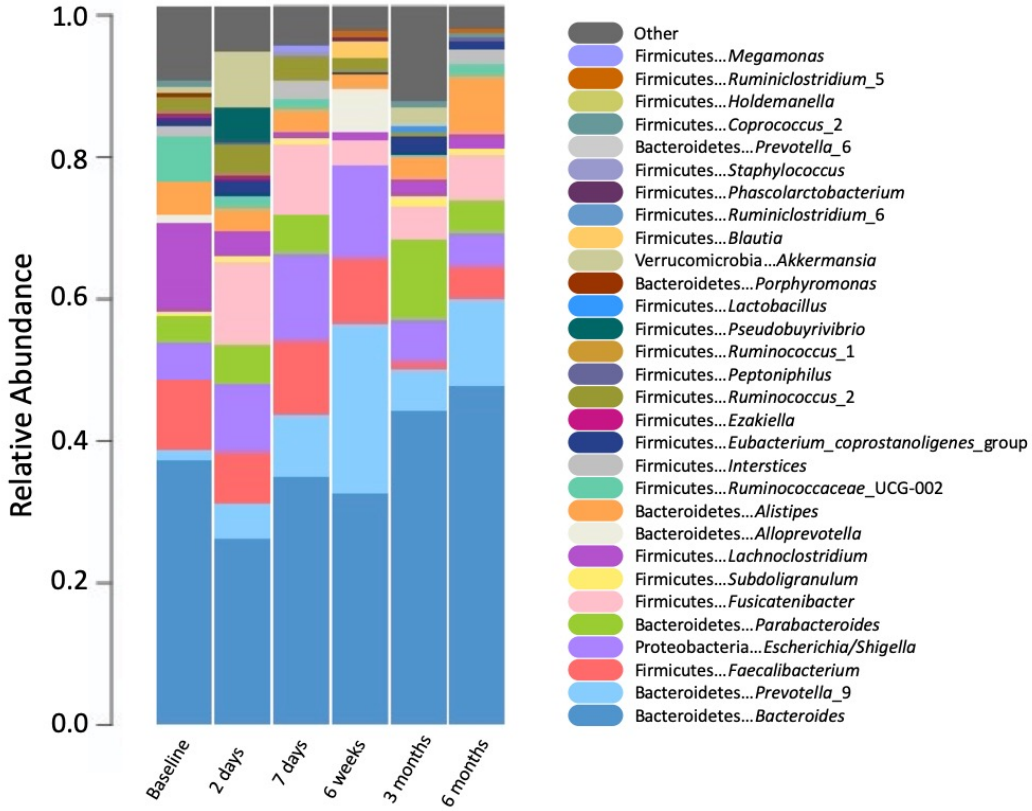
Supplementary Figure 5.18 Fecal microbiota composition of Patient 14 following an allogenic FMT. Patient 14 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



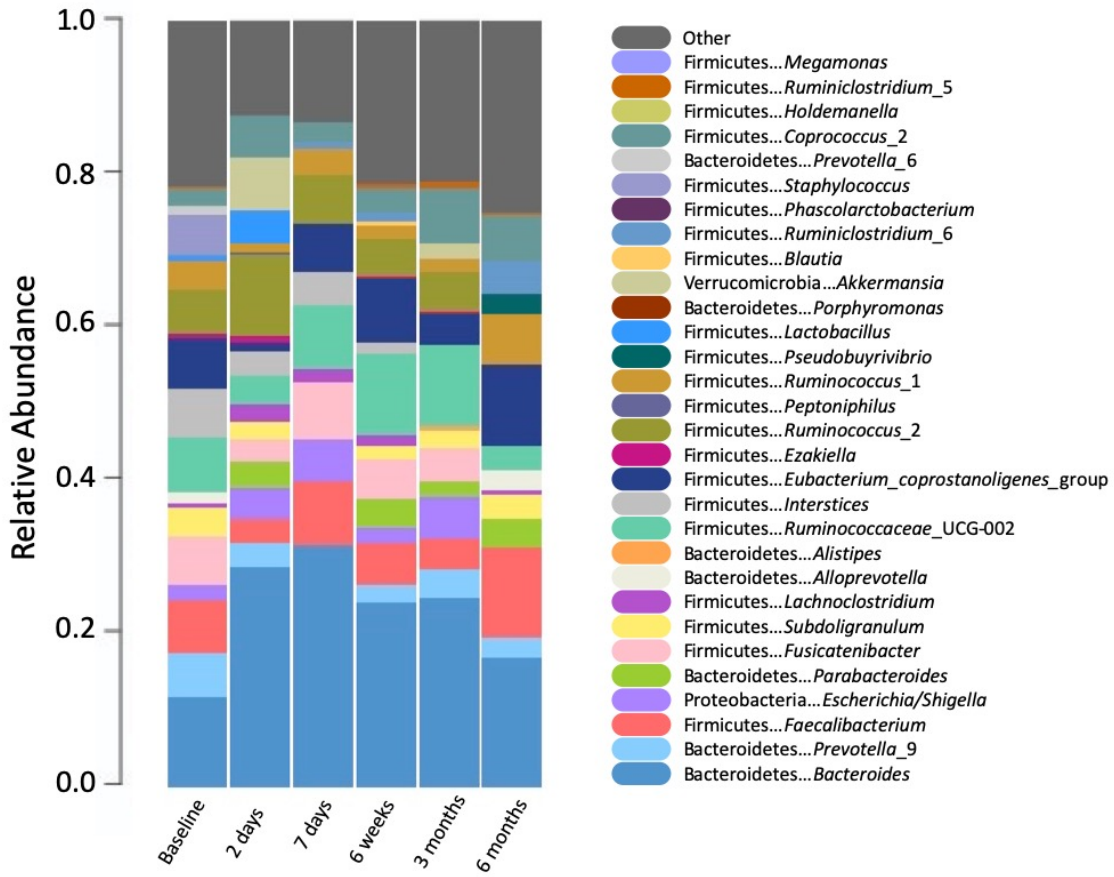
Supplementary Figure 5.19 Fecal microbiota composition of Patient 15 following an allogenic FMT. Patient 15 received an allogenic FMT from Donor 1. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



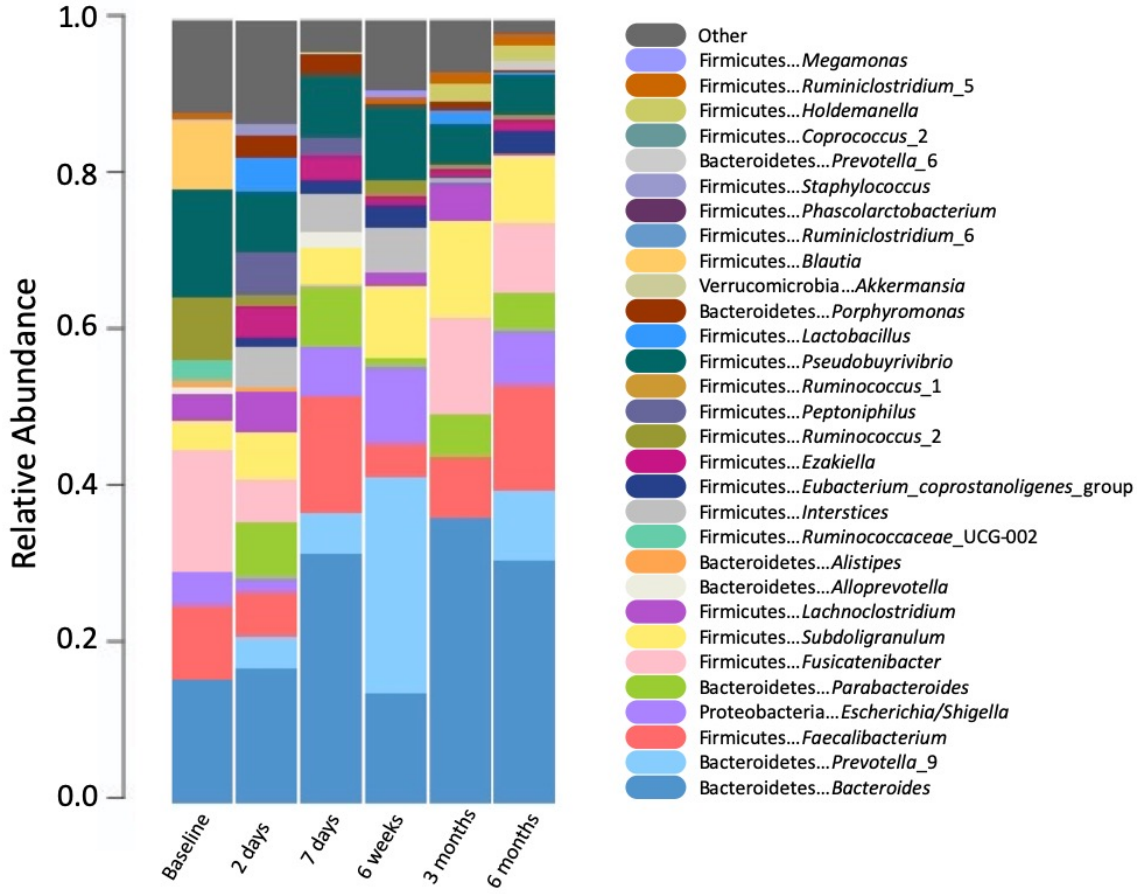
Supplementary Figure 5.20 Fecal microbiota composition of Patient 16 following an autologous FMT. Patient 16 received an autologous FMT. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



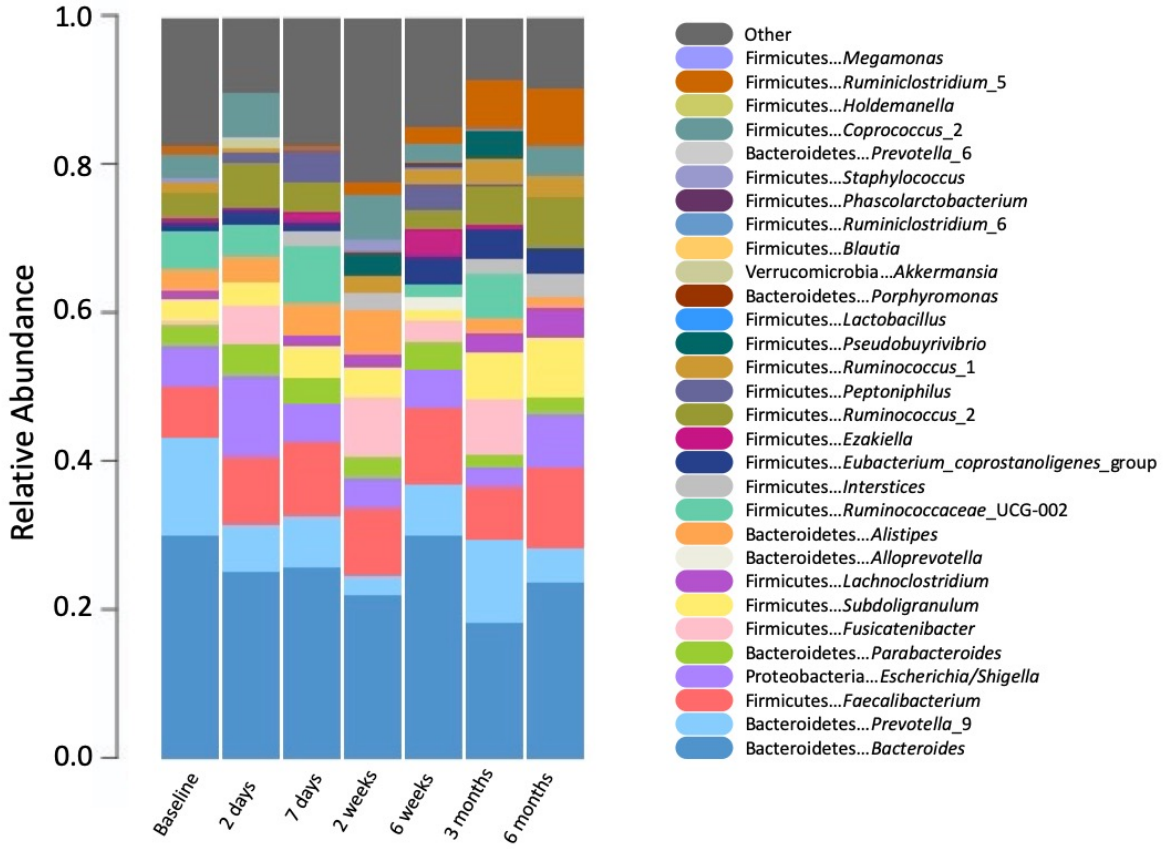
Supplementary Figure 5.21 Fecal microbiota composition of Patient 17 following an autologous FMT. Patient 17 received an autologous FMT. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



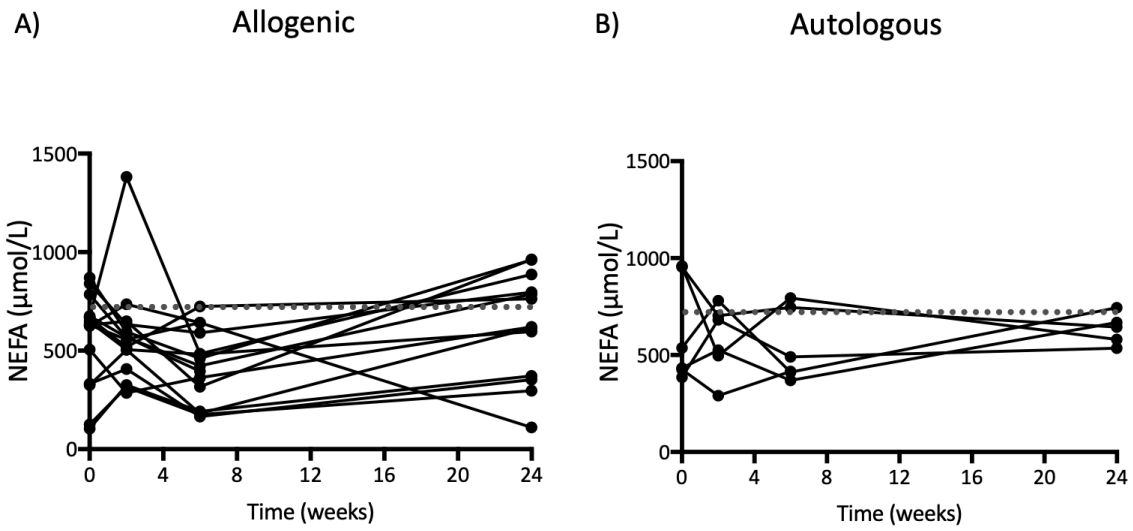
Supplementary Figure 5.22 Fecal microbiota composition of Patient 18 following an autologous. Patient 18 received an autologous FMT. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



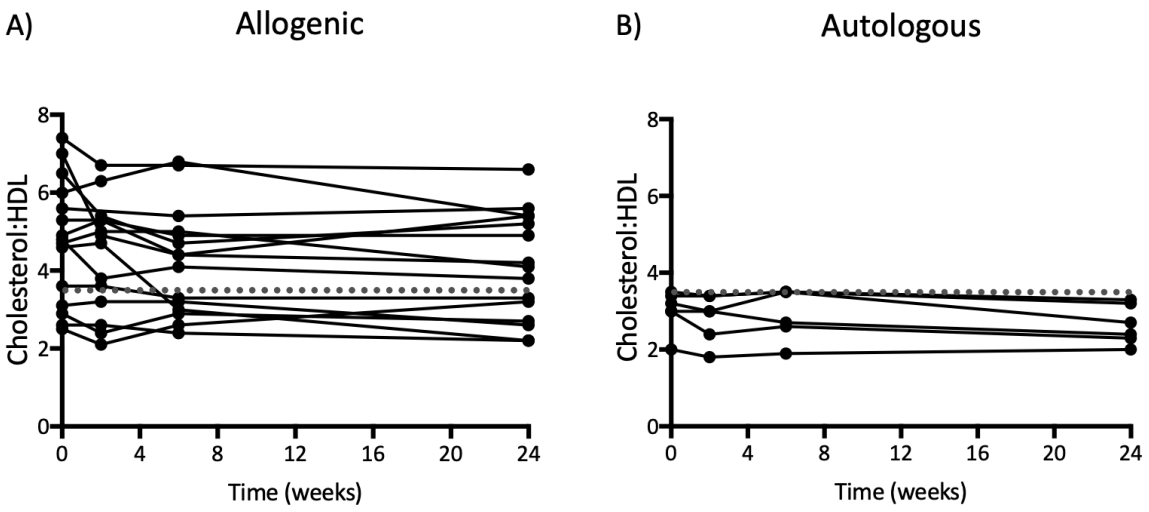
Supplementary Figure 5.23 Fecal microbiota composition of Patient 19 following an autologous FMT. Patient 19 received an autologous FMT. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



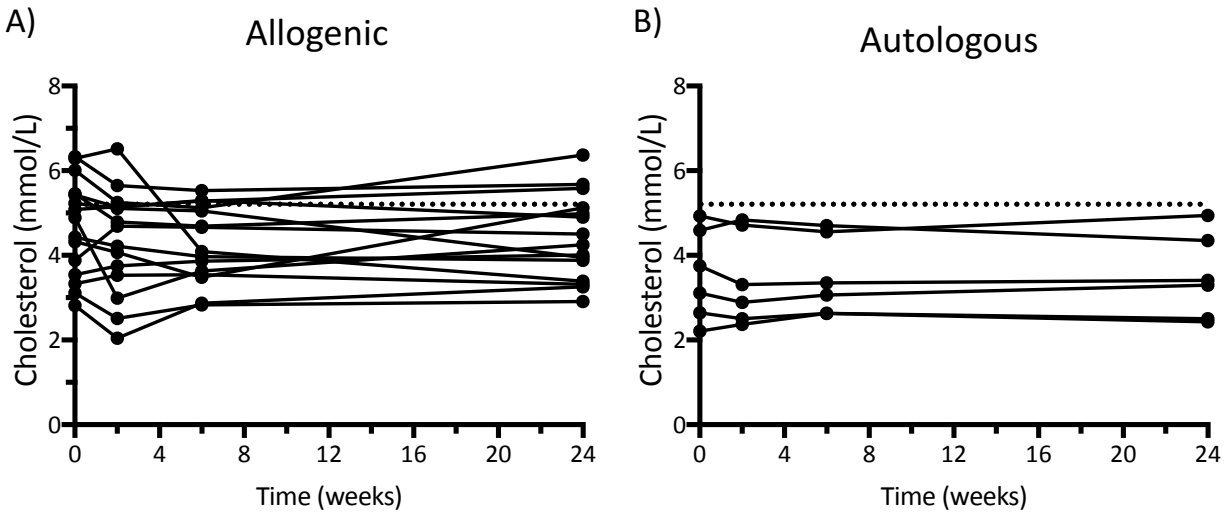
Supplementary Figure 5.24 Fecal microbiota composition of Patient 21 following an autologous FMT. Patient 21 received an autologous FMT. Toilet paper samples were collected from patients at baseline, 2 days, 7 days, 2 weeks, 6 weeks, 3 months, and 6 months post-FMT. The DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point.



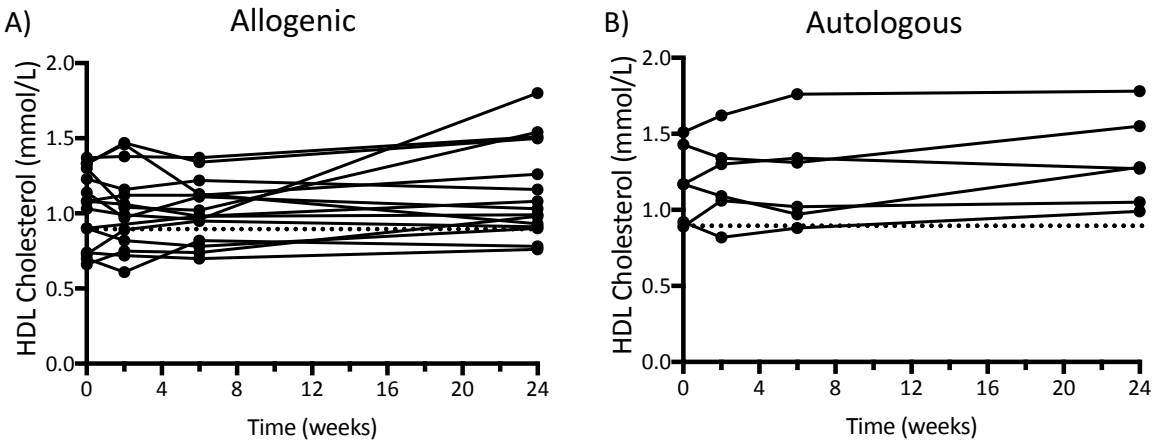
Supplementary Figure 5.25 Non-esterified fatty acids decrease at 6 weeks' post-transplant in patients who received an allogenic FMT. Non-esterified fatty acids were measured from blood samples taken at baseline, 2 weeks, 6 weeks, and 6 months. Wilcoxon matched-pairs signed rank test was performed to compare the NEFA concentrations at baseline and 6 weeks' post-FMT in both the allogenic and autologous groups (p=0.0084 and p=0.1562, respectively). A) The concentrations of non-esterified fatty acids of patients over time who received an allogenic FMT (n=15). Median (IQR); baseline: 631 µmol/L (332-675 µmol/L), 2 weeks: 557 µmol/L (407-633 µmol/L), 6 weeks: 423 µmol/L (192-591 µmol/L), and 6 months: 621 µmol/L (362.5-842 µmol/L). (B) The concentrations of non-esterified fatty acids of patients over time who received an autologous FMT (n=6). Median (IQR); baseline: 485 µmol/L (415-955.3 µmol/L), 2 weeks: 603.5 µmol/L (445.3-722.5 µmol/L), 6 weeks: 453 µmol/L (401.3-758 µmol/L), and 6 months: 644 (557.5-706 µmol/L). The red dotted line represents the cutoff for the normal concentration of NEFA, 720 µmol/L.



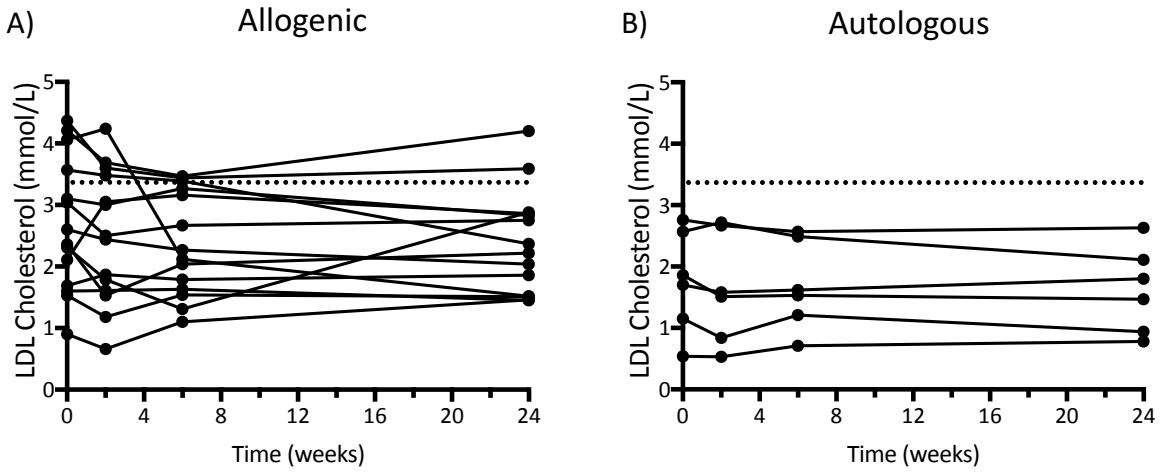
Supplementary Figure 5.26 Cholesterol:HDL ratio decreases over 6 months in patients who received an allogenic FMT. Cholesterol and HDL cholesterol were measured from blood samples taken at baseline, 2 weeks, 6 weeks, and 6 months. Wilcoxon matched-pairs signed rank test was performed to compare the Cholesterol:HDL ratio at baseline and 6 months' post-FMT in both the allogenic and autologous groups (p=0.0034 and p=0.0625, respectively). A) The ratios of Cholsterol:HDL of patients over time who received an allogenic FMT. Median (IQR); baseline: 4.8 (3.1-6), 2 weeks: 4.8 (3.05-5.325), 6 weeks: 4.4 (3-5), and 6 months 4.1 (2.7-5.4). B) The ratios of Cholesterol:HDL of patients over time who received an autologous FMT. Median (IQR); baseline: 3.1 (2.75-3.425), 2 weeks: 3 (2.25-3.4), 6 weeks: 3.1 (2.425-3.5), and 6 months: 2.55 (2.225-3.225). The red dotted line represents the cutoff for the normal ratio of Cholesterol:HDL, <3.7.



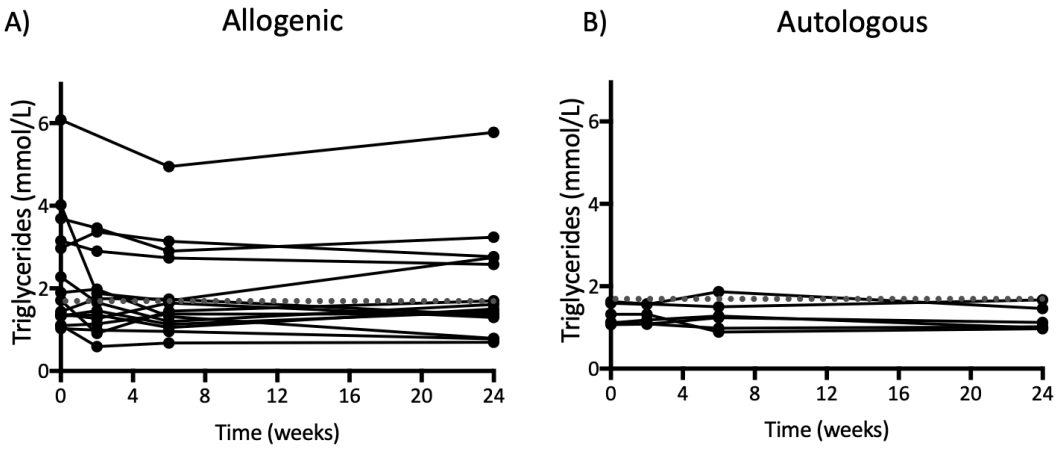
Supplementary Figure 5.27 The concentration of cholesterol does not change over time after FMT. Cholesterol was measured from blood samples taken at baseline, 2 weeks, 6 weeks, and 6 months. Wilcoxon matched-pairs signed rank test was performed to compare the cholesterol at baseline and 6 weeks' post-FMT in both the allogenic and autologous groups (p=0.0637 and p=0.844, respectively. A) The concentration of cholesterol over time in patients who received an allogenic FMT (n=15). B) The concentration of cholesterol over time in patients who received an autologous FMT (n=6). The black dotted line represents the cutoff for the normal concentration of cholesterol, 5.2 mmol/L.



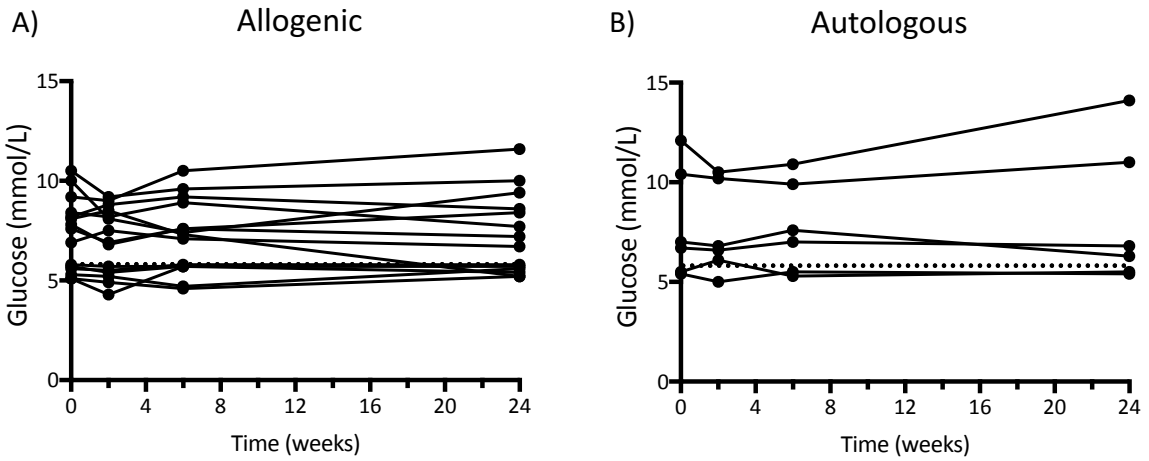
Supplementary Figure 5.28 The concentration of HDL cholesterol does not change over time after FMT. HDL cholesterol was measured from blood samples taken at baseline, 2 weeks, 6 weeks, and 6 months. Wilcoxon matched-pairs signed rank test was performed to compare the HDL cholesterol at baseline and 6 weeks' post-FMT in both the allogenic and autologous groups (p= 0.634 and p=0.688, respectively. A) The concentration of HDL cholesterol over time in patients who received an allogenic FMT (n=15). B) The concentration of HDL cholesterol over time in patients who received an autologous FMT (n=6). The black dotted line represents the cutoff for the normal concentration of HDL cholesterol, 0.9 mmol/L.



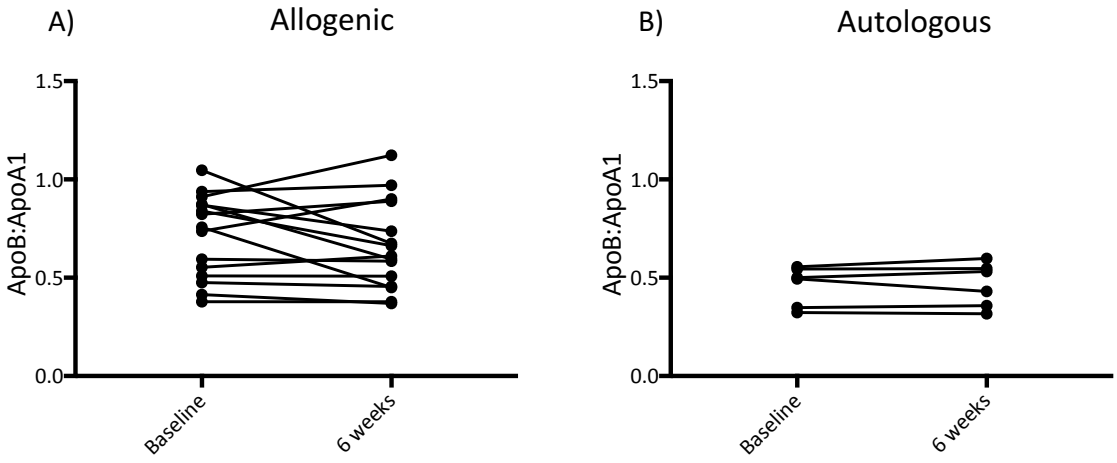
Supplementary Figure 5.29 The concentration of LDL cholesterol does not change over time after FMT. LDL cholesterol was measured from blood samples taken at baseline, 2 weeks, 6 weeks, and 6 months. Wilcoxon matched-pairs signed rank test was performed to compare the LDL cholesterol at baseline and 6 weeks' post-FMT in both the allogenic and autologous groups (p=0.153 and p=0.313, respectively. A) The concentration of LDL cholesterol over time in patients who received an allogenic FMT (n=15). B) The concentration of LDL cholesterol over time in patients who received an autologous FMT (n=6). The black dotted line represents the cutoff for the normal concentration of LDL cholesterol, 3.37 mmol/L.



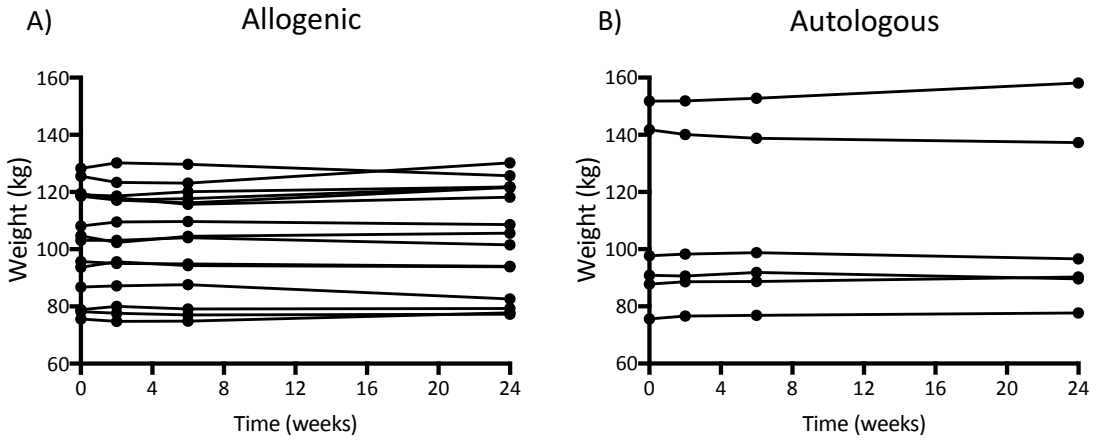
Supplementary Figure 5.30 The concentration of triglycerides is decreased at 6 weeks' post-FMT in after an allogenic FMT. Triglycerides was measured from blood samples taken at baseline, 2 weeks, 6 weeks, and 6 months. Wilcoxon matched-pairs signed rank test was performed to compare the triglycerides at baseline and 6 weeks' post-FMT in both the allogenic and autologous groups (p=0.027 and p=0.781, respectively). A) The concentration of triglycerides over time in patients who received an allogenic FMT (n=15). B) The concentration of triglycerides over time in patients who received an autologous FMT (n=6). The black dotted line represents the cutoff for the normal concentration of triglycerides, <1.7 mmol/L.



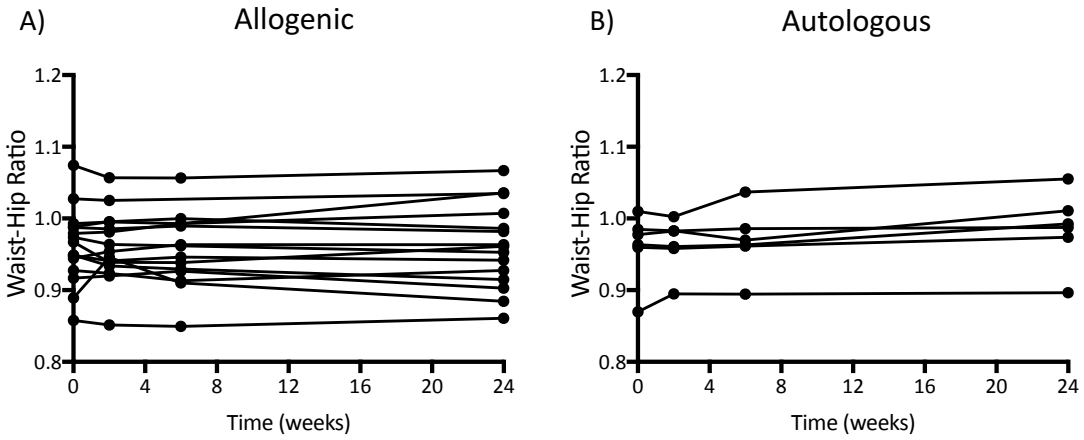
Supplementary Figure 5.31 The concentration of fasting glucose does not change over time after FMT. Fasting glucose was measured from blood samples taken at baseline, 2 weeks, 6 weeks, and 6 months. Wilcoxon matched-pairs signed rank test was performed to compare the fasting glucose at baseline and 6 weeks' post-FMT in both the allogenic and autologous groups (p=0.827 and p=0.844, respectively. A) The concentration of fasting glucose over time in patients who received an allogenic FMT (n=15). B) The concentration of fasting glucose over time in patients who received an autologous FMT (n=6). The black dotted line represents the cutoff for the normal concentration of glucose (fasting), 5.8 mmol/L.



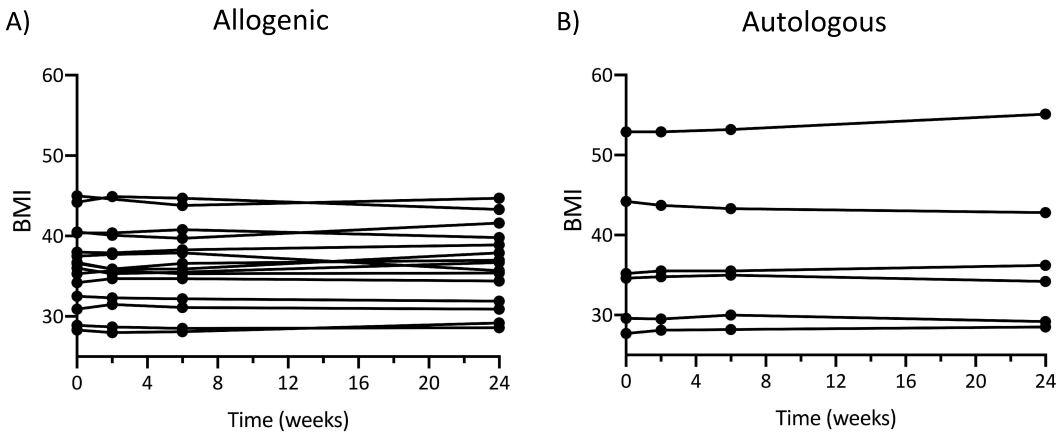
Supplementary Figure 5.32 Change in ApoB:ApoA1 over time. Concentrations of ApoB:ApoA1 were determined from fasting blood sample at baseline and 6 weeks' post-transplant. Wilcoxon matched-pairs signed rank test was performed to compare the ratio of ApoB:ApoA1 at baseline and 6 weeks' post-FMT in both the allogenic and autologous groups (p=0.330 and p=0.688, respectively). A) Ratio of ApoB:ApoA1 in patients who received an allogenic FMT over time (n=15). B) Ratio of ApoB:ApoA1 in patients who received an autologous FMT over time (n=6).



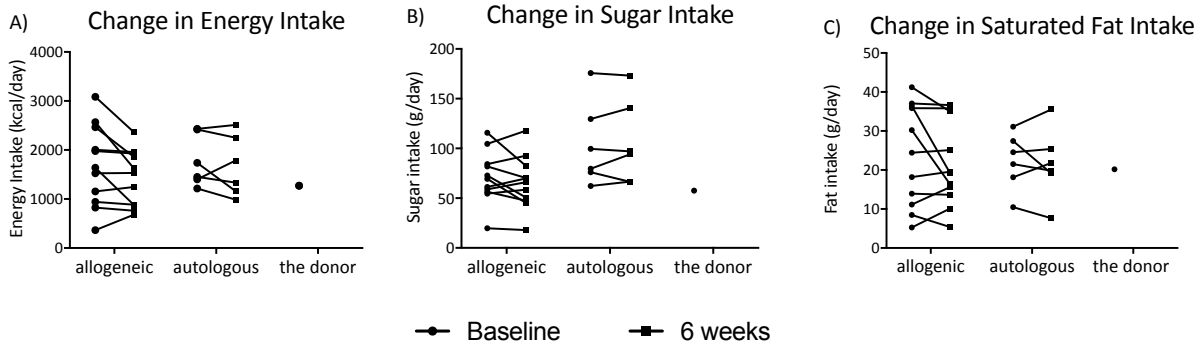
Supplementary Figure 5.33 Patients' weights do not change after receiving an FMT. Weight was measured at baseline, 2 weeks, 6 weeks and 6 months. A) The weights of patients over time who received an allogenic FMT (n=15). Median (IQR); baseline: 104.7 kg (86.8-119.1 kg), 2 weeks 102.8 kg (85.4-117.6 kg), 6 weeks: 104.5 kg (87.6-117.7 kg), and 6 months: 105.6 kg (82.6-121.8 kg). B) The weights of patients over time who received an autologous FMT (n=6). Median (IQR); baseline: 94.3 kg (84.75-144.3 kg), 2 weeks: 94.45 kg (85.6-143.1 kg), 6 weeks: 95.35 kg (85.75-142.3 kg), and 6 months: 93.45 kg (86.53-142.5 kg).



Supplementary Figure 5.34 Waist-to-hip ratio remains constant after an FMT. Waist and hip measurements were taken at baseline, 2 weeks, 6 weeks, and 6 months. A) The waist-to-hip ratios of patients over time who received an allogenic FMT (n=15). Median (IQR); baseline: 0.9673 (0.9274-0.9884), 2 weeks: 0.9537 (0.9337-0.9553), 6 weeks: 0.954 (0.9232-0.9929), and 6 months: 0.9613 (0.915-1.0070). B) The waist-to-hip ratios of patients who received an autologous FMT (n=6). Median (IQR); baseline: 0.9706 (0.9274-0.9912), 2 weeks: 0.9717 (0.9424-0.9877), 6 weeks: 0.9664 (0.9448-0.9986), and 6 months: 0.9901 (0.9544-1.022).



Supplementary Figure 5.35 BMI remains constant after FMT. Weight and height were measured at baseline, 2 weeks, 6 weeks, and 6 months. A) The BMI of patients who received an allogenic FMT (n=15). Median (IQR); baseline: 36.6 (32.5-40.4), 2 weeks: 35.8 (32.1-38.5), 6 weeks: 35.9 (32.2-39.7), and 6 months: 36.7 (31.9-39.8). B) The BMI of patients who received an autologous FMT (n=6). Median (IQR); baseline: 34.9 (29.1-46.4), 2 weeks: 35.2 (29.2-46.0), 6 weeks: 35.3 (29.6-45.8), and 6 months: 35.2 (29.0-45.9).



Supplementary Figure 5.36 Nutrient and caloric intake do not change following allogenic and autologous FMT. Patients completed DHQII at baseline and 6 weeks post-FMT. Students' t-tests were used to compare the differences in changes in intakes at baseline and 6 weeks post-FMT in both the allogenic and autologous groups. There were 7 participants at baseline with energy intakes <1500; 4 in the allogenic group and 3 in the autologous group this is likely to represent underreporting of energy intakes. At follow up there were 8 under reporters; 5 allogenic and 3 autologous. Under reporters were included. There was no statistically significant between group difference in reduction in energy intakes allogenic 260±410kcal/ day vs autologous 100±320kcal/ day (p=0.45).

Appendix D – Chapter 6 Supplementary Material

Supplementary Table 6.1. Baseline and change from baseline in chemistry data

Laboratory Test		6 FMTs (n=6)
Albumin	**Baseline mean ± SD**	45.0 ± 1.0
	Change in mean after treatment	0.17
	***P*-value**	0.750
AlkPhos	**Baseline mean ± SD**	42.3 ± 7.4
	Change in mean after treatment	-5.5
	***P*-value**	0.500
ALT	**Baseline mean ± SD**	20.4 ± 12.5
	Change in mean after treatment	1.6
	***P*-value**	0.625
AST	**Baseline mean ± SD**	20.0 ± 3.3
	Change in mean after treatment	-1.2
	***P*-value**	0.625
Anion Gap	**Baseline mean ± SD**	12.2 ± 1.9
	Change in mean after treatment	0.13
	***P*-value**	>0.999
Bicarbonate	**Baseline mean ± SD**	27.4 ± 1.5
	Change in mean after treatment	-1.73
	***P*-value**	0.438
Bilirubin, Total	**Baseline mean ± SD**	11.8 ± 8.0
	Change in mean after treatment	0.4
	***P*-value**	0.563
Calcium	**Baseline mean ± SD**	2.25 ± 0.06
	Change in mean after treatment	0.025
	***P*-value**	0.188
Chloride	**Baseline mean ± SD**	100.8 ±1.3
	Change in mean after treatment	0.5
	***P*-value**	0.625
Creatinine	**Baseline mean ± SD**	67.6 ± 16.0
	Change in mean after treatment	3.9
	***P*-value**	0.125
Glucose, Random	**Baseline mean ± SD**	4.4 ± 0.7
	Change in mean after treatment	0.5
	***P*-value**	0.250
Magnesium	**Baseline mean ± SD**	0.88 ± 0.07
	Change in mean after treatment	-0.008
	***P*-value**	0.250
Potassium	**Baseline mean ± SD**	3.8 ± 0.2

	Change in mean after treatment	0.22
	***P*-value**	0.438
Sodium	**Baseline mean ± SD**	139.6 ± 2.7
	Change in mean after treatment	0.8
	***P*-value**	0.750
Urea	**Baseline mean ± SD**	4.74 ± 1.78
	Change in mean after treatment	-0.47
	***P*-value**	0.688

Wilcoxon matched-pairs signed rank test used for statistical analysis.

Supplementary Table 6.2 Baseline and change in baseline in hematology data

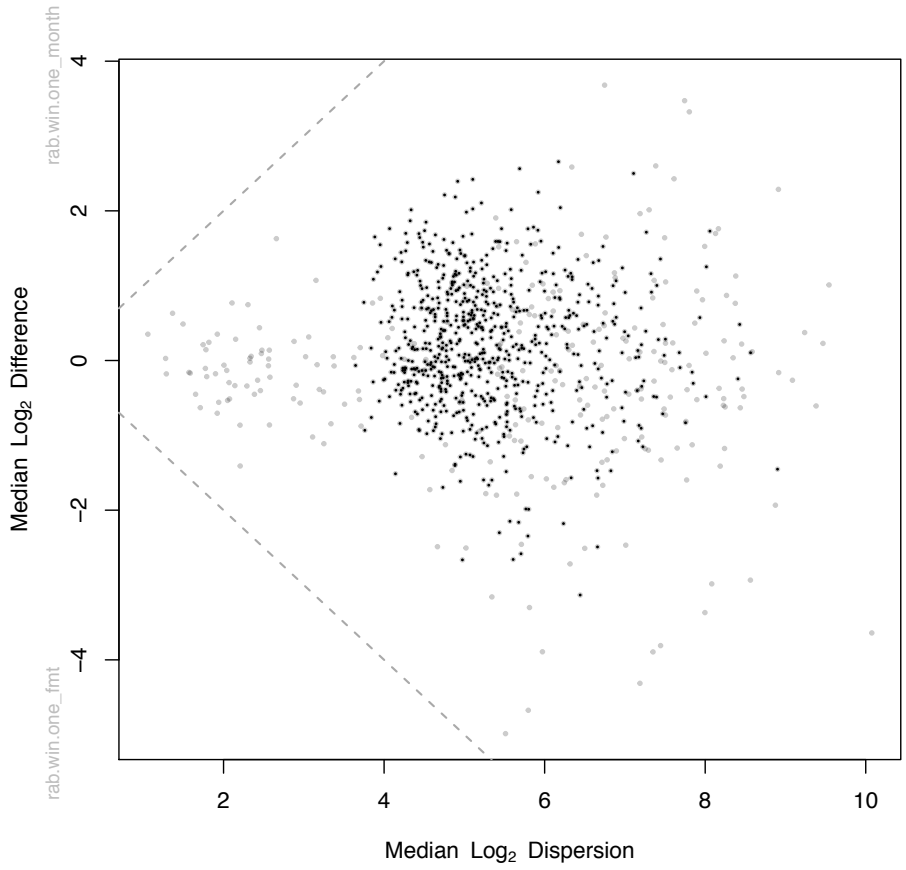
Laboratory Test		6 FMTs (n=6)
LKC	**Baseline mean ± SD**	6.1 ± 1.5
	Change in mean after treatment	-0.7
	***P*-value**	>0.999
ERC	**Baseline mean ± SD**	4.55 ± 0.40
	Change in mean after treatment	-0.025
	***P*-value**	0.500
Hemoglobin	**Baseline mean ± SD**	140 ± 8
	Change in mean after treatment	-1
	***P*-value**	0.125
HCT	**Baseline mean ± SD**	0.42 ± 0.02
	Change in mean after treatment	0.00
	***P*-value**	>0.999
MCV	**Baseline mean ± SD**	92.1 ± 5.6
	Change in mean after treatment	0.9
	***P*-value**	0.188
RDW CV	**Baseline mean ± SD**	12.8 ± 0.4
	Change in mean after treatment	-0.3
	***P*-value**	0.375
MPV	**Baseline mean ± SD**	10.3 ± 2.0
	Change in mean after treatment	0.5
	***P*-value**	0.250
Thrombocytes	**Baseline mean ± SD**	206 ± 60
	Change in mean after treatment	2
	***P*-value**	0.813
Neutrophils	**Baseline mean ± SD**	3.9 ± 1.5
	Change in mean after treatment	-0.5
	***P*-value**	>0.999
Lymphocyte	**Baseline mean ± SD**	1.5 ± 0.4
	Change in mean after treatment	-0.1
	***P*-value**	>0.999
Monocyte	**Baseline mean ± SD**	0.5 ± 0.2
	Change in mean after treatment	0.0
	***P*-value**	>0.999
Eosinophil	**Baseline mean ± SD**	0.1 ± 0.1
	Change in mean after treatment	0.0
	***P*-value**	>0.999
Basophil	**Baseline mean ± SD**	0.0
	Change in mean after treatment	0.0
	***P*-value**	>0.999

Wilcoxon matched-pairs signed rank test used for statistical analysis.

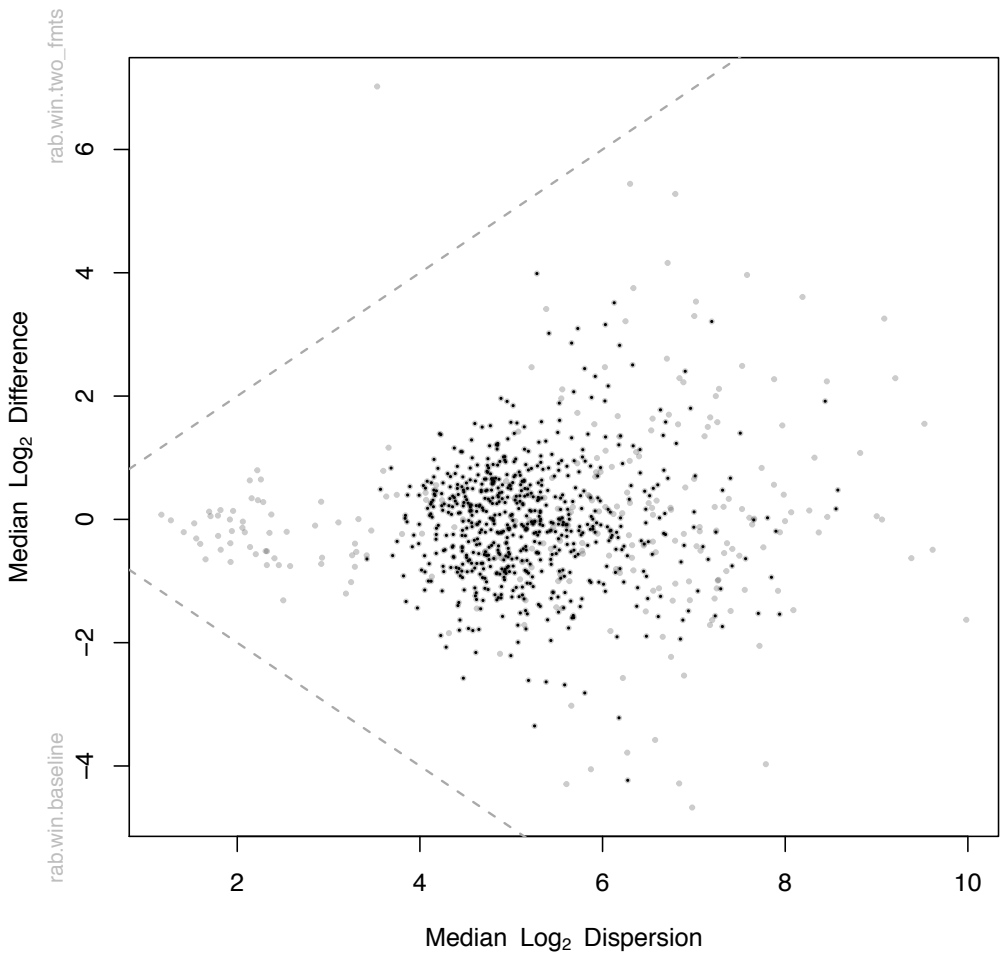
Table 6.3 Baseline and change from baseline in vital signs.

Assessment		6 FMTs (n=6)
Heart rate (bpm)	**Baseline mean ± SD**	73 ± 7
	Change in mean after treatment	4
	***P*-value**	0.469
Systolic pressure (mmHg)	**Baseline mean ± SD**	111 ± 15
	Change in mean after treatment	11
	***P*-value**	0.0625
Diastolic pressure (mmHg)	**Baseline mean ± SD**	71 ± 6
	Change in mean after treatment	6
	***P*-value**	0.0625
Respiration rate (breaths per min)	**Baseline mean ± SD**	16.3 ± 0.8
	Change in mean after treatment	0.3
	***P*-value**	>0.999
Oral Temperature (° C)	**Baseline mean ± SD**	36.3 ± 0.4
	Change in mean after treatment	0
	***P*-value**	0.438

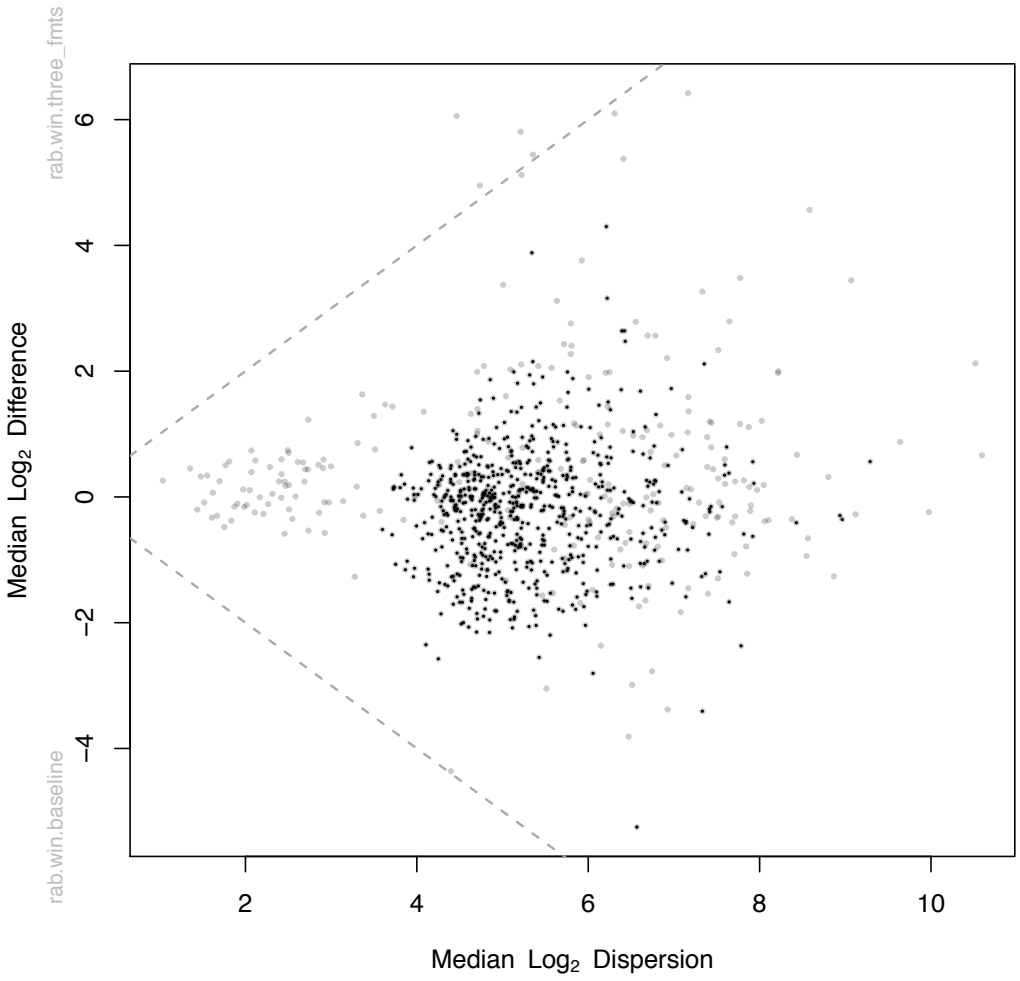
Wilcoxon matched-pairs signed rank test used for statistical analysis.



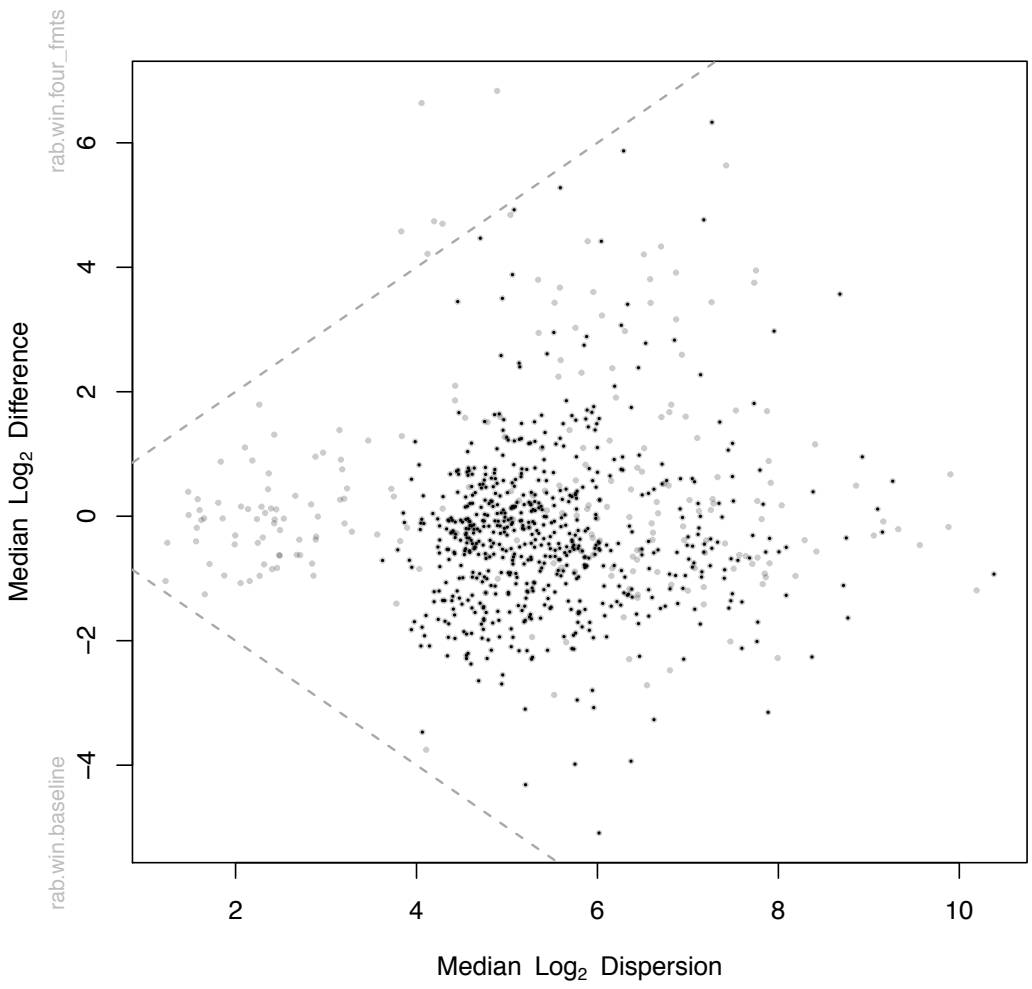
Supplementary Figure 6.1. ALDEx plot of MS patients at baseline compared to 1 month (1 FMT). Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of MS patients at baseline compared to receiving one FMT. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red.



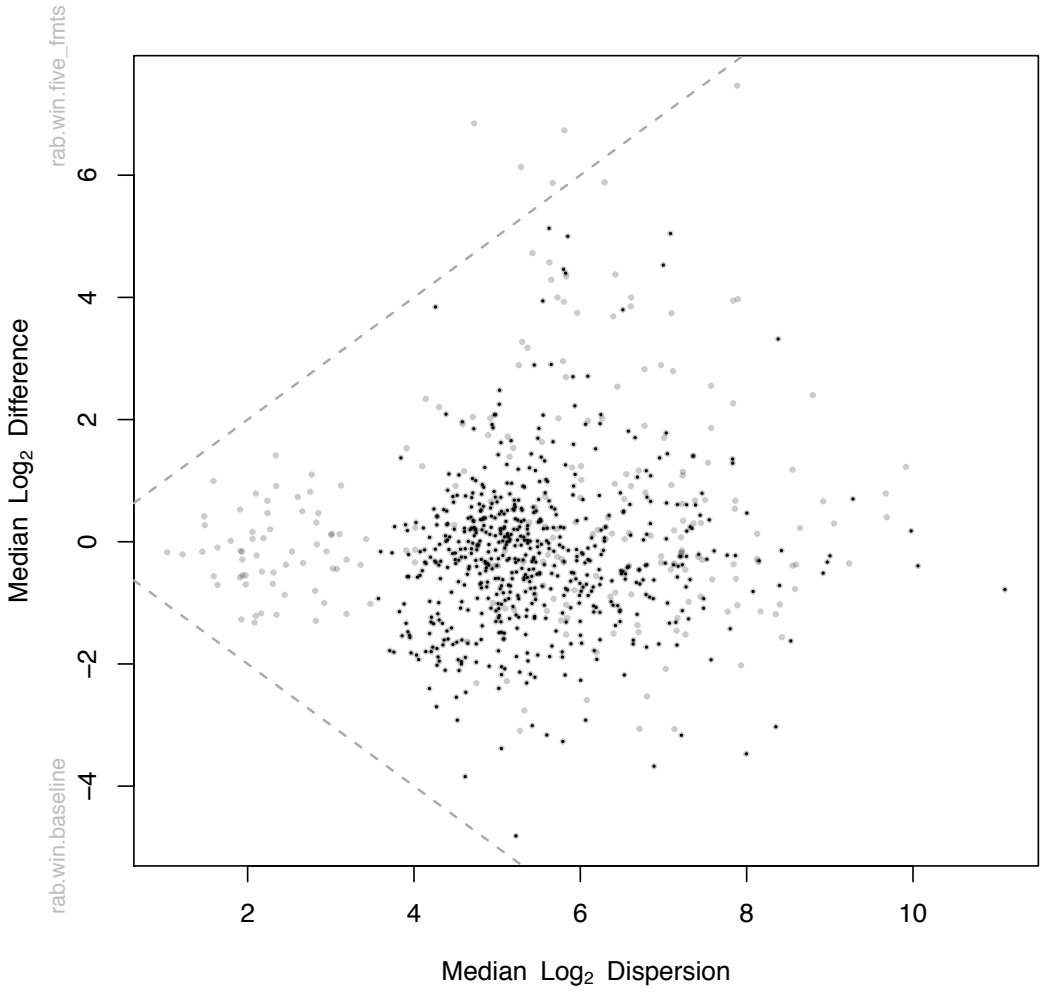
Supplementary Figure 6.2 ALDEx plot of MS patients at baseline compared to 2 months (2 FMTs). Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of MS patients at baseline compared to receiving two FMTs. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red.



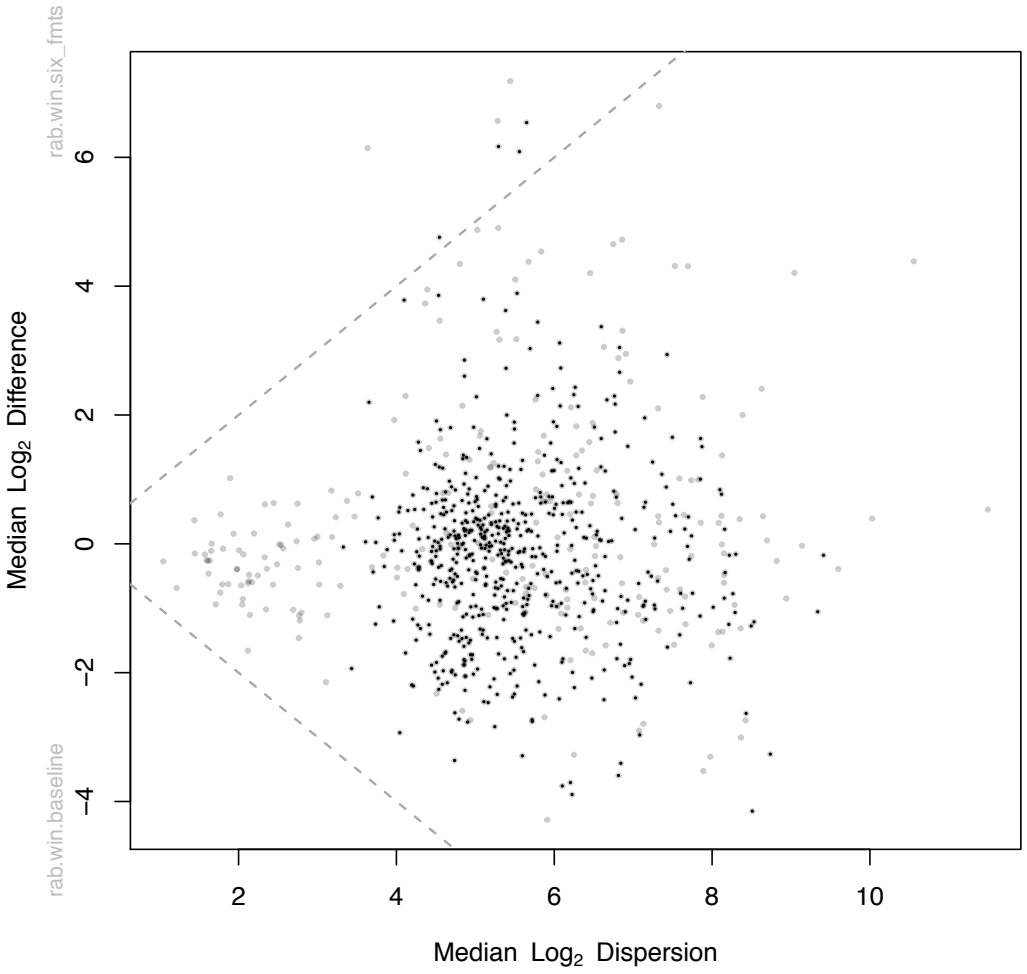
Supplementary Figure 6.3 ALDEx plot of MS patients at baseline compared to 3 months (3 FMTs). Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria in MS at baseline compared to receiving 3 FMTs. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red.



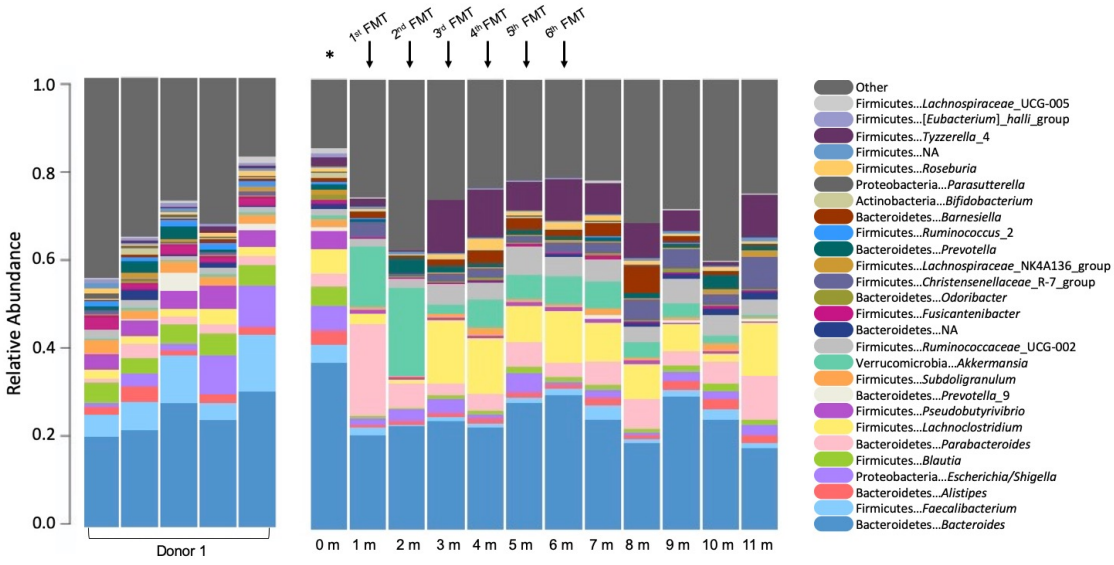
Supplementary Figure 6.4 ALDEx plot of MS patients at baseline compared to 4 months (4 FMTs). Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of MS patients at baseline compared to receiving four FMTs. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red.



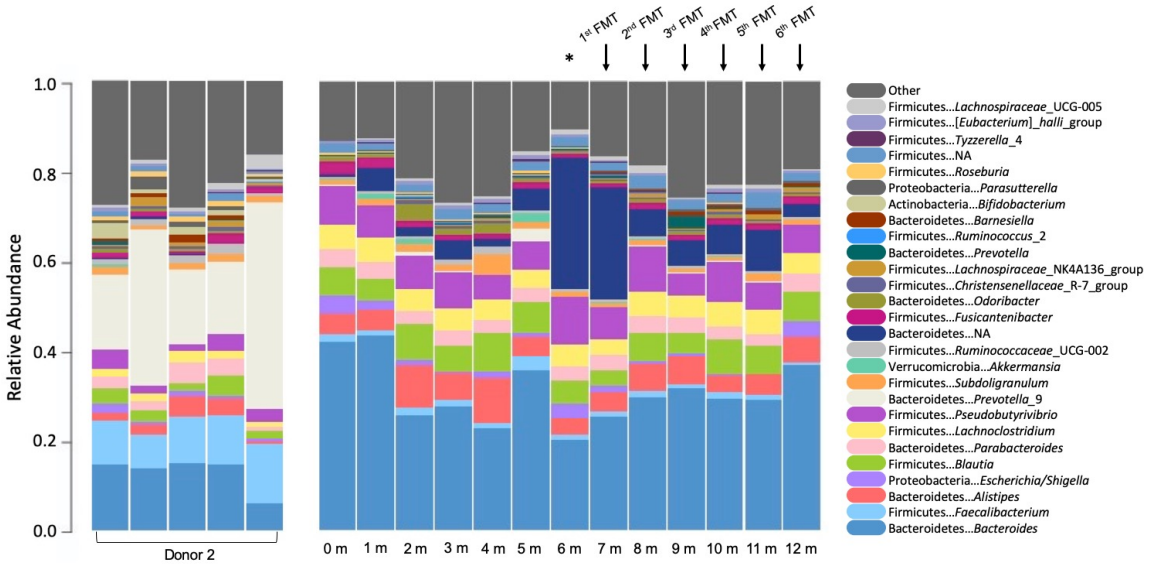
Supplementary Figure 6.5 ALDEx plot of MS patients at baseline compared to 5 months (5 FMTs). Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of MS patients at baseline compared to receiving five FMTs. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red.



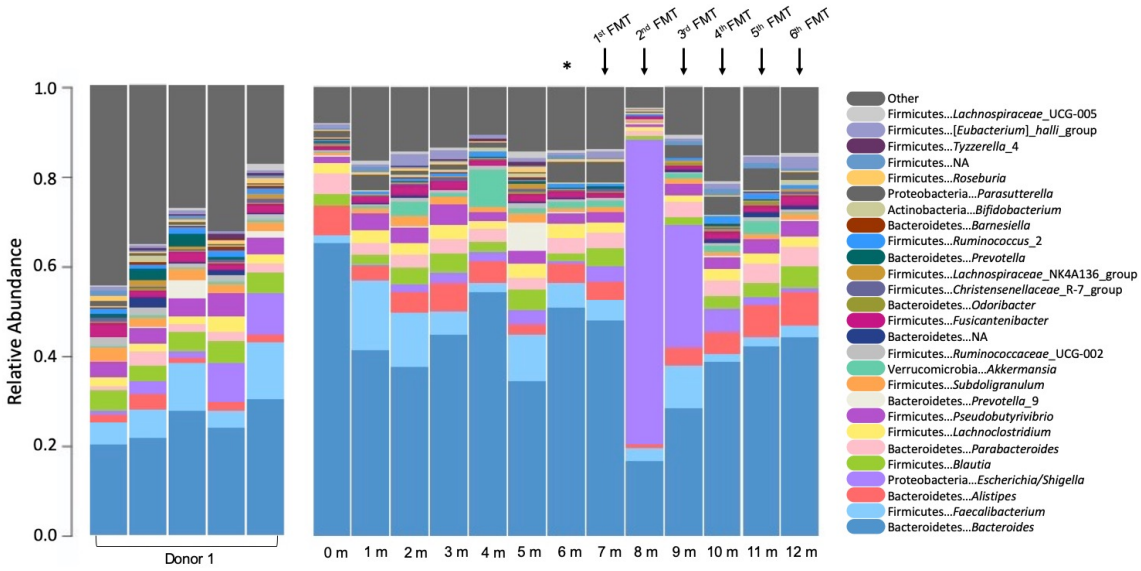
Supplementary Figure 6.6 ALDEx plot of MS patients at baseline compared to 6 months (6 FMTs). Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were analyzed using ALDEx2 (version 3.9) in R (version 3.5.3) to identify differentially abundant genera. There were no significant changes in the composition of fecal bacteria of MS patients at baseline compared to receiving six FMTs. Non-significant genera that are rare are shown in black and non-significant genera that are abundant are shown in grey. Significant genera would be shown in red.



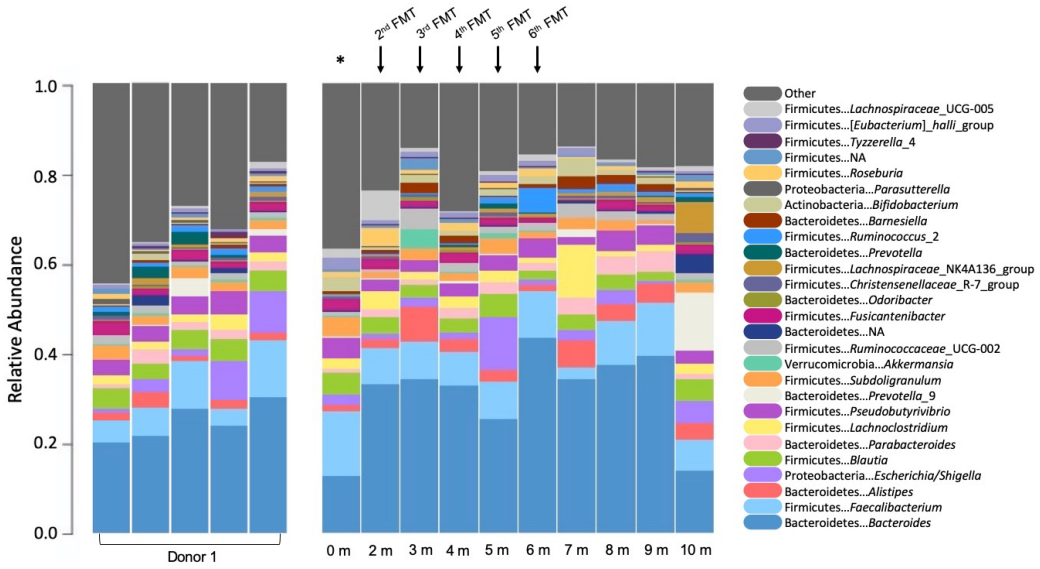
Supplementary Figure 6.7 Fecal microbiota composition of patient MK-FMT-001. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 11 months in the early intervention group; they received six FMTs from donor 1 and were followed for six months following the last FMT. Black arrows indicate the appointments that the patient received an FMT. * indicates the baseline microbiota, taken 2-3 days before the first FMT was administered.



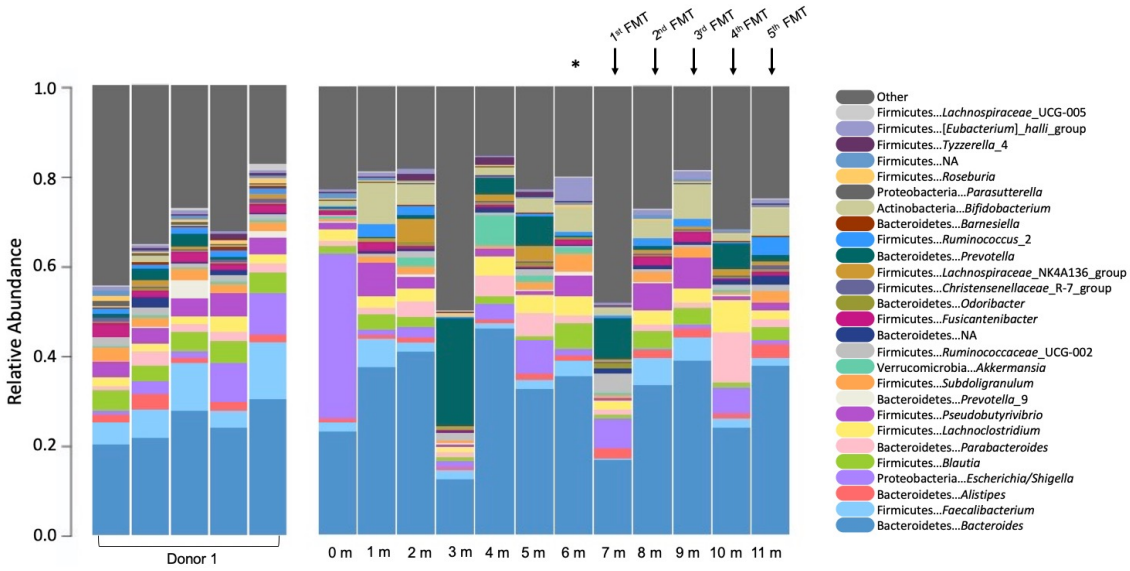
Supplementary Figure 6.8 Fecal microbiota composition of patient MK-FMT-002. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 12 months in the late intervention group; they received six FMTs from donor 2. Black arrows indicate the appointments that the patient received an FMT. * indicates the baseline microbiota, taken 2-3 days before the first FMT was administered.



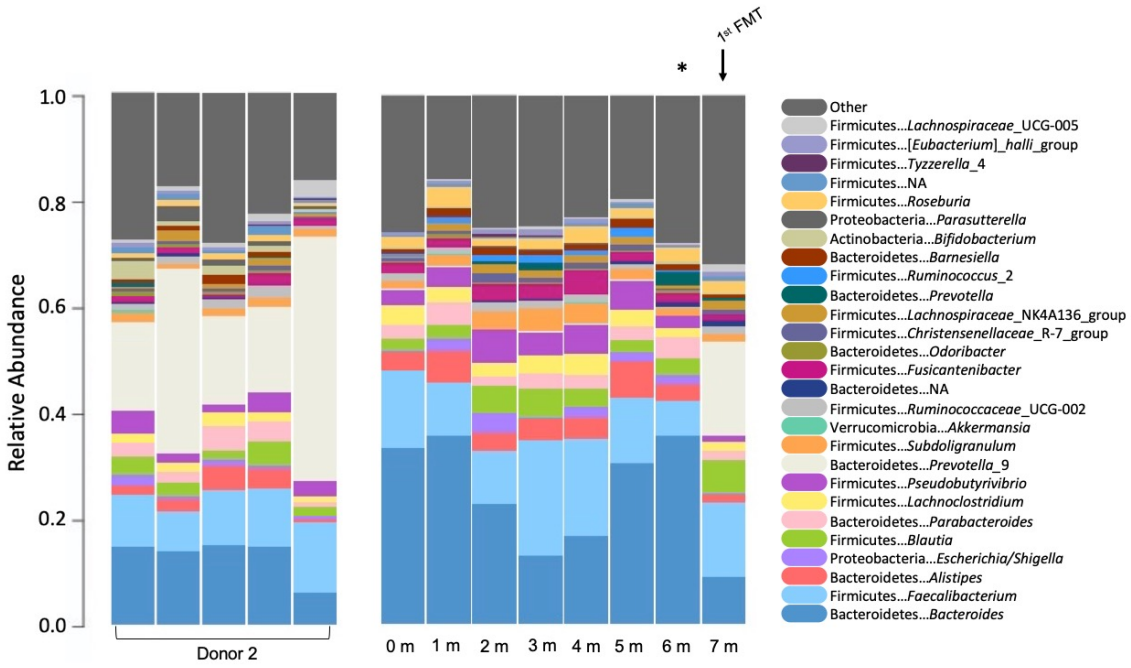
Supplementary Figure 6.9 Fecal microbiota composition of patient MK-FMT-003. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 12 months in the late intervention group; they received six FMTs from donor 2. Black arrows indicate the appointments that the patient received an FMT. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.



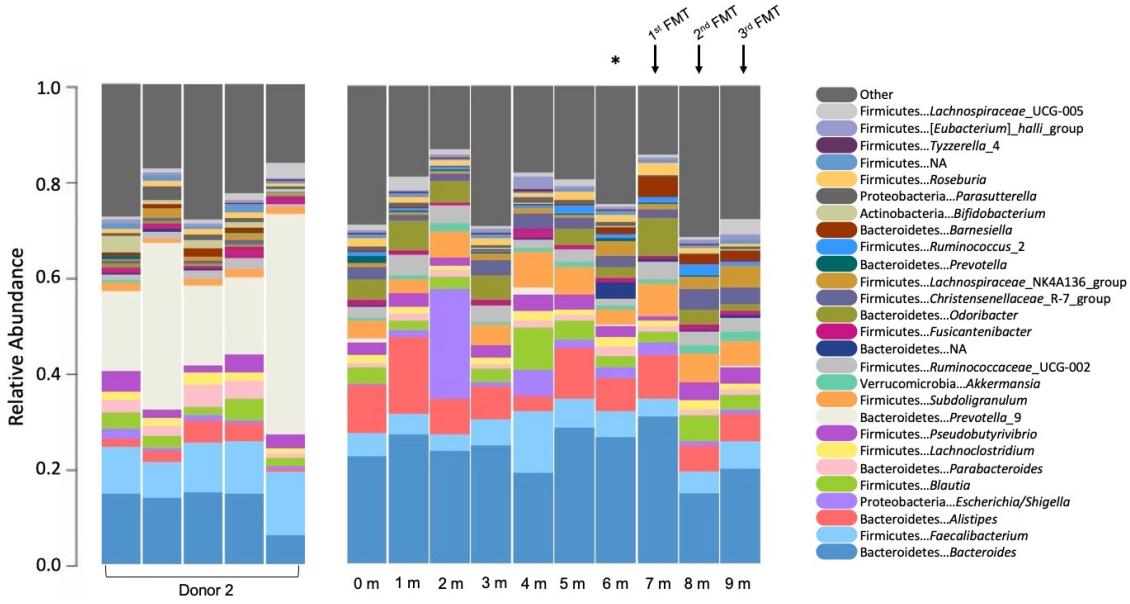
Supplementary Figure 6.10 Fecal microbiota composition of patient MK-FMT-004. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 10 months in the early intervention group; they received six FMTs from donor 1. Black arrows indicate the appointments that the patient received an FMT. This patient forgot to bring in a fecal sample at the 1 month time point. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.



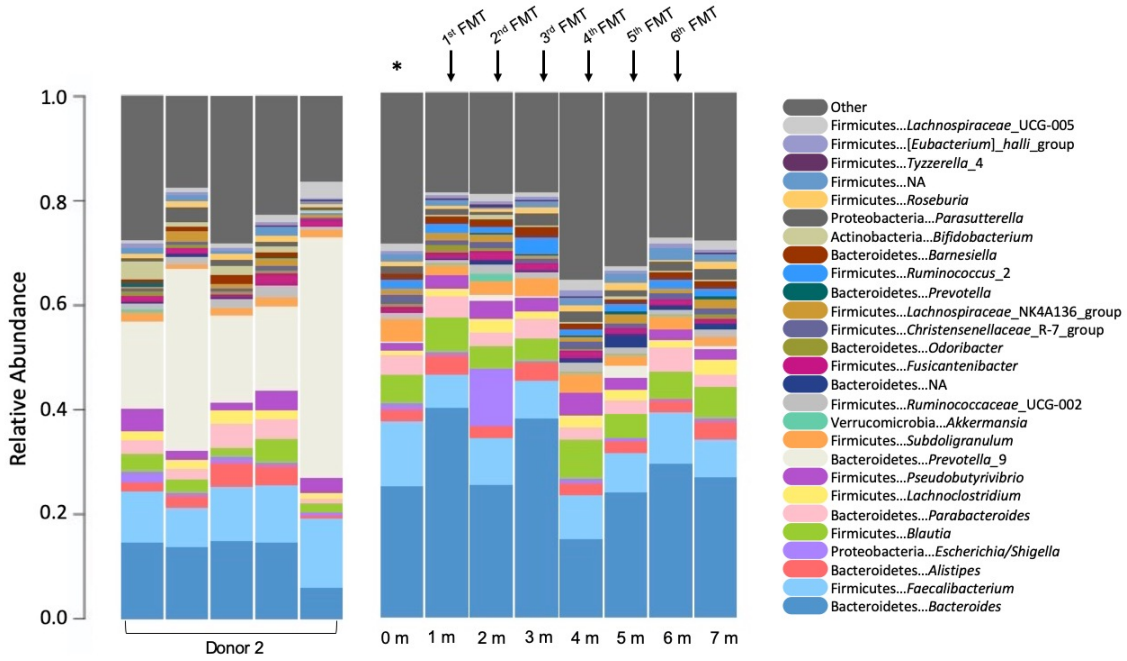
Supplementary Figure 6.11 Fecal microbiota composition of patient MK-FMT-007. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 12 months in the late intervention group; they received six FMTs from donor 1. Black arrows indicate the appointments that the patient received an FMT. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.



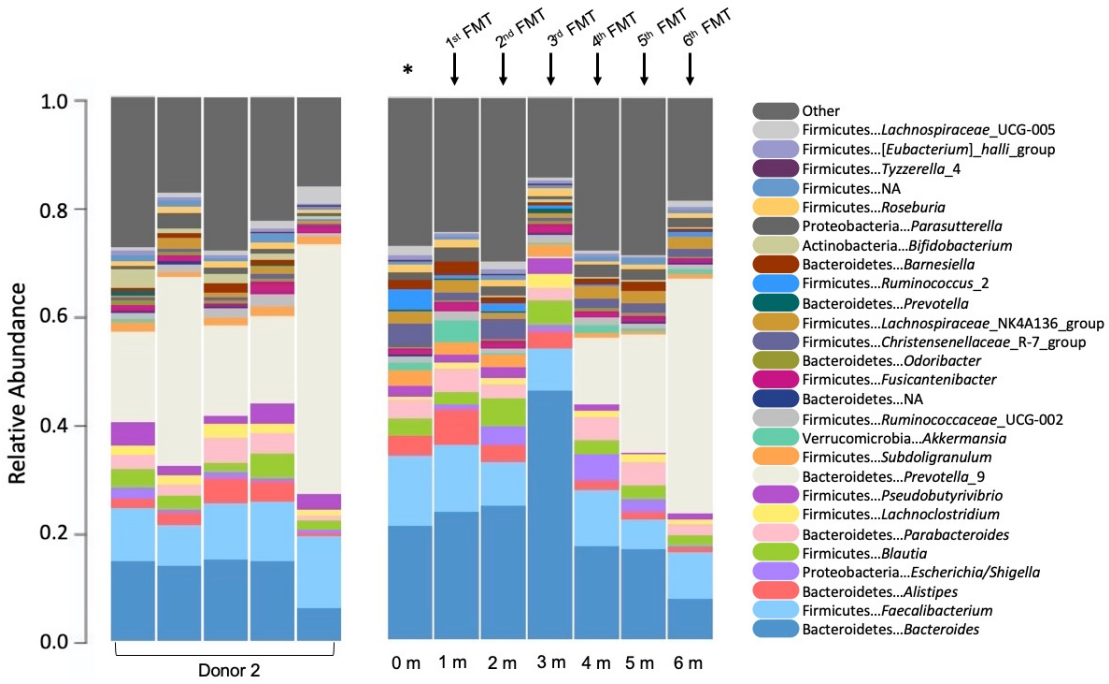
Supplementary Figure 6.12 Fecal microbiota composition of patient MK-FMT-008. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 7 months in the late intervention group; they received two FMTs from donor 2. Black arrows indicate the appointments that the patient received an FMT. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.



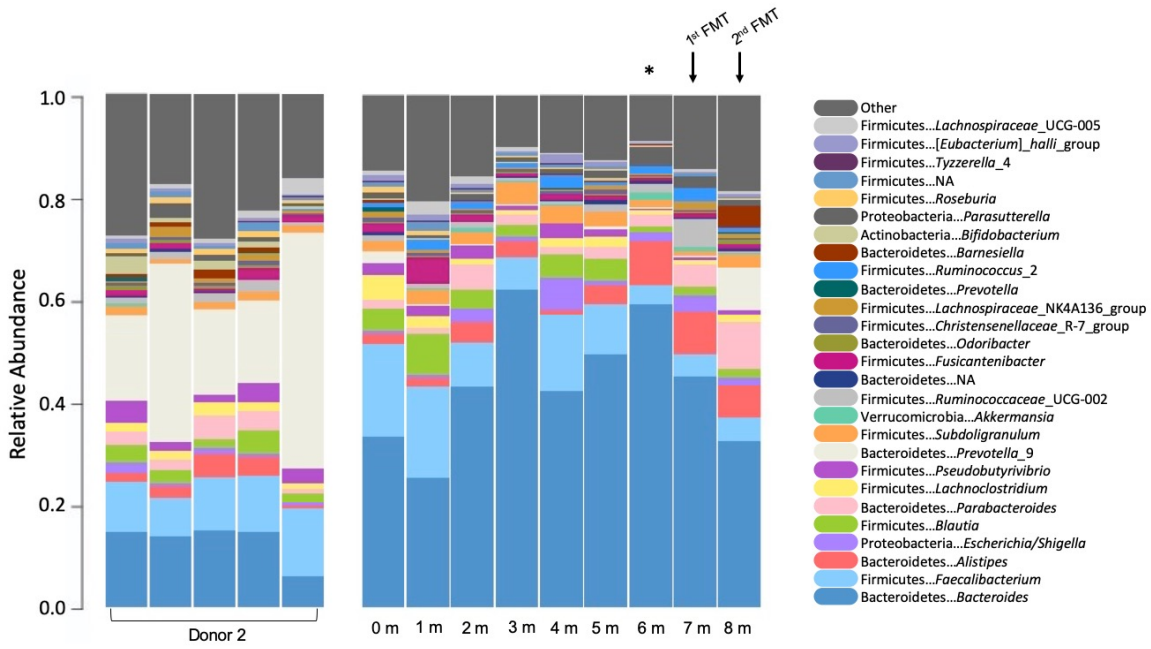
Supplementary Figure 6.13 Fecal microbiota composition of patient MK-FMT-009. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 9 months in the late intervention group; they received four FMTs from donor 2. Black arrows indicate the appointments that the patient received an FMT. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.



Supplementary Figure 6.14 Fecal microbiota composition of patient MK-FMT-010. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 7 months in the early intervention group; they received six FMTs from donor 1. Black arrows indicate the appointments that the patient received an FMT. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.



Supplementary Figure 6.15 Fecal microbiota composition of patient MK-FMT-011. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 6 months in the early intervention group; they received six FMTs from donor 2. Black arrows indicate the appointments that the patient received an FMT. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.



Supplementary Figure 6.16 Fecal microbiota composition of patient MK-FMT-012. Fecal samples were collected from patients one to two days before their scheduled appointments, the DNA was extracted and the V4 region of the 16S rRNA gene was amplified and sent for Illumina MiSeq next-generation sequencing. The resulting reads were used to generate bar plots in base R (version 3.5.3). Each column represents the fecal microbiota composition at one time point. This patient was in the study for 8 months in the late intervention group; they received three FMTs from donor 2. Black arrows indicate the appointments that the patient received an FMT. * indicate the baseline microbiota, taken 2-3 days before the first FMT was administered.

Curriculum Vitae

Name: Laura Craven

Post-secondary Education and Degrees:
Western University
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BMSc (Microbiology and Immunology), with distinction
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Honours and Awards:
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2018, 2019

Best Poster Award (ISAPP Chicago, IL)
2017

Dean's Honor List
2012, 2013, 2014, 2015

Related Work Experience
Teaching Assistant (MicroImm 4100A and MicroImm 3610E)
Western University
September 2017- December 2019

Student Representative (Lawson Association of Students and Fellows)
Lawson Health Research Institute
May 2016 – April 2019

Student Representative
London Health Research Day
January 2018 – April 2019

Publications:

Craven LJ, Rahman A, Parvathy SN, et al. Allogenic fecal microbiota transplantation in patients with non-alcoholic fatty liver disease improves abnormal small intestinal permeability: a randomized control trial. *American Journal of Gastroenterology* (Accepted March 29th, 2020).

Chen JM, Al KF, **Craven LJ,** et al. Nutritional, microbial, and allergenic changes during the fermentation of cashew 'cheese' product using a quinoa-based rejuvelac starter culture. *Nutrients*. **12**; 648 (2020). Doi: 10.3390/nu12030648

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Craven L, Silverman MS, Burton JP. Transfer of altered behaviour and IBS-D through fecal microbiota transplant in mouse model indicates need for stricter donor screening criteria. *Annals of Translational Medicine*. **5**; (2017). doi: 10.21037/atm.2017.10.03

Presentations and Posters:

Craven L, Rahman A, Nair Parvathy S, Beaton M, Silverman J, Qumosani K, Hramiak I, Hegele RA, Joy T, Meddings J, Urqhart BL, Harvie R, McKenzie CA, Summers K, Reid G, Burton JP, Silverman MS. Fecal microbiota transplantation in patients with non-alcoholic fatty liver disease and metabolic syndrome has the potential to improve small intestinal permeability. Poster: Resident Research Day, London, ON, May 10th, 2019.

Craven L. Expanding the use of fecal microbiota transplants beyond *C. difficile* infection and their future. Presentation: International Scientific Association for Probiotics and Prebiotics, Chicago, IL, June 28th, 2017.

Craven L, Gloor G, Reid G, Silverman MA, Burton JP. What are the optimal long-term storage conditions for donor samples used in fecal microbiota transplantation? Poster: International Scientific Association for Probiotics and Prebiotics, Chicago, IL, June 28th, 2017.

Craven L, Nair Parvathy S, Tat-Ko J, Burton JP, Silverman MS. Extended screening costs associated with selecting donors for fecal microbiota transplantation for treatment of metabolic syndrome-associated diseases. Poster: American Society of Microbiology, New Orleans, LA, June 3rd, 2017.

Craven L. Fecal microbiota transplantations and their therapeutic use in treating non-alcoholic fatty liver disease. Presentation: Talks on Fridays, London, ON, February 26th, 2016.